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(71) Applicants *(for all designated States except US)*: LABORATOIRES OM S.A. [CH/CH]; 22, route du Bois du Lan, CH-1217 Meyrin (CH). DEUTSCHE OM ARZNEIMITTEL GMBH [DE/DE]; Am Houiller Platz 17, D-61381 Friedrichsdorf (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DAVIES, John, Gwynfor [GB/CH]; 163, route de Collex, CH-1239 Collex-Bossy (CH). BAUER, Jacques [CH/CH]; 31, chemin de la Moraine, CH-1162 Saint-Prex (CH). HIRT, Pierre [CH/CH]; 64D, route de Genève, CH-1028 Préverenges (CH). SCHULTHESS, Adrian [CH/CH]; En Loye, CH-1268 Begnins (CH).

(74) Agent: PRINS, Hendrik, Willem; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

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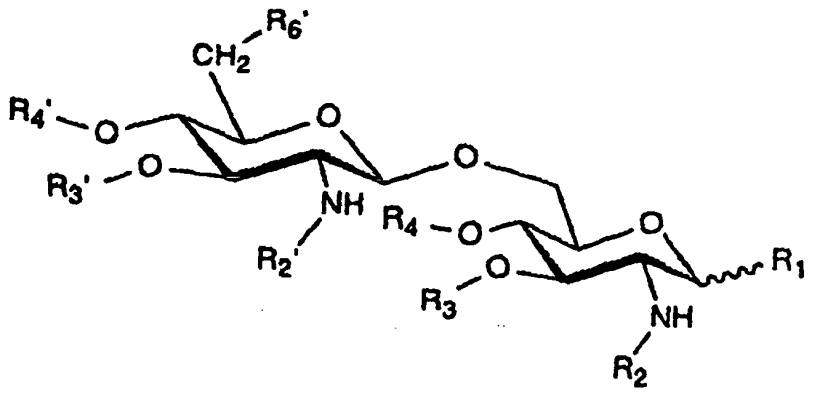

(I)

(57) Abstract

The invention relates to β(1→6) glucosamine disaccharides having general formula (I) to a method for preparing these disaccharides, comprising the steps of: i) providing a starting material comprising lipid A moiety of lipopolysaccharide-comprising microorganisms; and ii) subjecting the starting material to an alkaline treatment such that lipid A moiety is O-deacylated at the 3-position and at the 3'-position, to pharmaceutical compositions comprising as an active ingredient these disaccharides, and to these disaccharides for use as an immunomodulating agent, anti-tumor agent, and vaccine component.

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Glucosamine disaccharides, method for their preparation, pharmaceutical composition comprising same, and their use

The present invention relates to specific glucosamine disaccharides, in particular to 2-N- and/or 2'-N-acylated glucosamine disaccharides, wherein at least one of the acyl groups is branched, and to compounds comprising these disaccharides. The present invention relates further to methods for preparing these disaccharides from starting materials comprising the lipid A moiety of lipopolysaccharides which starting material is subjected to a specific alkaline treatment. The invention relates also to pharmaceutical compositions comprising these disaccharides as an active ingredient, and finally to the use of these disaccharides in therapy and prophylaxis.

Lipopolysaccharides constitute endotoxins of micro-organisms such as Gram-negative bacteria, and comprise a polysaccharide component and a lipid component. This lipid component, also called lipid A, determines the endotoxic properties of lipopolysaccharides (Rietschel E. Th. et al. in Immunobiology, Volume 186, pages 169-190 [1993]).

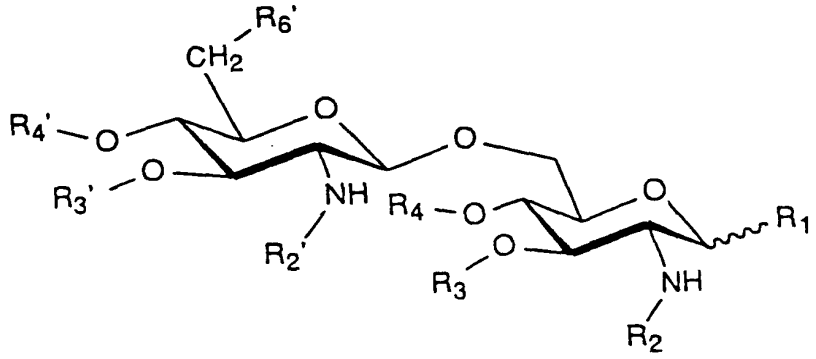
In US-A-4,912,094 modified lipopolysaccharides have been disclosed which exhibit reduced endotoxic properties while maintaining their antigenic and immuno-stimulating properties. These modified lipopolysaccharides are 3-O-deacylated and may be converted into 3-O-deacylated disaccharides by acid hydrolysis. Of these compounds the monophosphoryl 3-O-deacylated disaccharide is less toxic than the diphosphoryl 3-O-deacylated disaccharide.

The present invention relates to disaccharides which are 3-O-deacylated and 3'-O-deacylated or comprise at the 3-O-position and/or the 3'-O-position a short O-linked alkyl or acyl group, and comprise at least an N-linked, branched acyl group at the 2-position, 2'-position, or at both the

2-position and 2'-position. These compounds exhibit a still lower endotoxicity while maintaining biological activity (such as immunomodulation) and possess anti-cancer activity.

It was surprising that these specific glucosamine disaccharides possess the combination of lower endotoxicity and maintained biological activity, because although synthetic 3-O- and 3'-O-deacylated glucosamine disaccharides comprising an N-linked acyl group at the 2- and 2'-position (compound 307, Takada, H. et al. in CRC Critical Reviews in Microbiology, Volume 16, pages 477-523 [1989]; and compound LA-19-PP, Rietschel et al. [1993]) exhibited some immunobiological activities in in vitro assays, these activities were far weaker than those of reference bacterial lipid A specimens. They also lacked typical endotoxic activity.

Accordingly, the present invention relates to ß(1→6) glucosamine disaccharides having the general formula



wherein

R_1 is a hydroxyl group,
a dihydroxyphosphonoyloxy group or its charged forms,
a (C_1-C_5)acyloxy group;
a (C_1-C_5)alkyloxy group, or
a group X;

R_2 and R_2' are each an acyl group or a group Y with the proviso that at least R_2 or R_2' is the group Y;

R_3 and R_3' are each hydrogen,
a (C_1-C_3)alkyl group, or
a (C_1-C_3)acyl group;

R_4 is hydrogen,
a (C_1-C_3)alkyl group, or
a (C_1-C_3)acyl group;

R_4' is hydrogen,
a (C_1-C_5)acyl group,
a (C_1-C_5)alkyl group,
a dimethoxyphosphonoyl group, or
a phosphono group or its charged forms; and

R_6' is hydrogen,
a hydroxyl group,
a dihydroxyphosphonoyloxy group,
a hydroxysulphonyloxy group, their charged forms,
or a group Z;

wherein the group X is selected from the group comprising
a carboxy (C_1-C_5)alkyloxy group;
an $-O-CH-[(CH_2)_mCOOH][(CH_2)_nCOOH]$ group,
wherein m = 0-5 and
n = 0-5;
a phosphono(C_1-C_5)alkyl group;
a dimethoxyphosphonoyloxy group;
a hydroxysulphonyloxy group;
a hydroxysulphonyl(C_1-C_5)alkyl group; and
charged forms of the group X;

wherein the group Y is selected from the group comprising
an acyloxyacyl group,
an acylaminoacyl group,
an acylthioacyl group,
a (C_1-C_{24})alkyloxyacyl group,
a (C_1-C_{24})alkylaminoacyl group,
a (C_1-C_{24})alkylthioacyl group; and

wherein the group Z is selected from the group comprising
a (C_1-C_{24})alkyloxy group;
a (C_1-C_{24})acyloxy group;
3-deoxy-D-manno-2-octulosonic acid (KDO);
$(KDO)_n$, wherein n = 1-10;
a polysaccharide side chain, such as a side chain
originating from natural lipopolysaccharide;
a core component, such as a component originating
from natural lipopolysaccharide; and

amino-(C_1-C_8)alkyl-carboxyl group;

and its salts.

These glucosamine disaccharides exhibit a far lower endotoxicity, determined in the limulus amoebocyte lysate (LAL) test, than lipopolysaccharides (LPS) from for instance E.coli, lipid A and modified lipid A according to US-A-4,912,094. Furthermore, these glucosamine disaccharides according to the invention induce nitric oxide reactive intermediates and cytokines, such as interleukin 1-alpha (IL 1-alpha), IL-6, tumor necrosis factor (TNF) and prostaglandin (PGE).

In addition, these disaccharides show anti-tumor activity such as in peritoneal carcinomatosis.

Finally the acute toxicity of these disaccharides is extremely low. No deaths were monitored in Swiss mice after an intravenous dose of 100 mg disaccharide per kg body weight.

The present invention relates also to a method for preparing these glucosamine disaccharides using starting material from biological origin, that is, any starting material comprising the lipid A moiety of polysaccharides from micro-organisms, such as Gram-negative bacteria. According to the invention this starting material is subjected to at least an alkaline treatment such that the sugar O-linked acyl and/or O-linked oxyacyl groups are removed. If appropriate, the alkaline-treated starting material may be subjected to further treatments for removing the polysaccharide and core component (by acid treatment), and for changing or exchanging the substituents at the 1-position, 2-position, 3-position, 4-position, 2'-position, 3'-position, 4'-position and 6'-position.

However, the glucosamine disaccharides according to the invention can also be obtained by synthesis starting from the corresponding glucosamine disaccharide and introduce the objective substituents at the 2- and/or 2'-position.

Due to the extremely low endotoxicity in combination with the above-disclosed biological activity, these disaccharides according to the invention form an elite active

ingredient of a pharmaceutical composition. Such a pharmaceutical composition and the disaccharides per se may be used as immunomodulating agent, anti-tumor agent and as a vaccine component.

The glucosamine disaccharide according to the invention (a ß(1→6) D-glucosamine dimer) is characterized in that each glucosamine comprises at the 3- and 3'-position a hydroxyl group or a short O-linked alkyl or acyl group not substantially changing endotoxicity and/or biological activity, and further at least one N-linked, branched acyl group at the 2- or 2'-position or at both 2- and 2'-position. The remaining 2'- or 2-position is N-acylated. Presumably the presence of two hydrophobic chains at the 2-position and the 2'-position, at least one of which is in the form of a branched acyl group, imparts the disaccharide with the combination of extremely low endotoxicity and maintained biological activity.

The branched acyl group, herein in general referred to as the group Y is selected from the group comprising an acyloxyacyl group, an acylaminoacyl group, an acylthioacyl group, a (C_1-C_{24})alkyloxyacyl group, a (C_1-C_{24})alkylaminoacyl group, and a (C_1-C_{24})alkylthioacyl group.

In the case of the acyloxyacyl group, the two acyl groups are linked via an oxygen atom, in the case of the acylaminoacyl group via an NH group, and in the case of the acylthioacyl group via a sulphur atom. The other members of the group Y, the (C_1-C_{24})alkyloxyacyl group, the (C_1-C_{24})alkylaminoacyl group and the (C_1-C_{24})alkylthioacyl group may be obtained starting from the corresponding hydroxy fatty acid.

Preferably, the group Y represents an N-linked acyl group branched at its 3-position, such as a 3-acyloxyacyl group, a 3-acylaminoacyl group, and the 3-acylthioacyl group. The same applies to the aforementioned (C_1-C_{24})alkyl equivalents.

Preferably the members of the group Y comprise one or two acyl moieties, preferably selected from fatty acid residues, hydroxy fatty acid residues and oxy fatty acid

residues. When the acyloxyacyl group is preferably a 3-acyloxyacyl group, these acyl moieties comprise a 3-hydroxy fatty acid residue or for the ester-linked group a 3-oxo fatty acid residue. Typical examples of the acyloxyacyl group are 3-hydroxy(C_4-C_{24})-fatty acid-acyls which are ester-linked at the 3-hydroxy position with a (C_1-C_{24})-carboxylic acid. Preferably the acyloxyacyl group is a 3-hydroxy(C_8-C_{18})-fatty acid-acyl which is ester-linked at the 3-hydroxy position with (C_{10}-C_{18})-fatty acid. Such acyloxyacyl groups are present in the lipid A component of Gram-negative bacteria, such as Escherichia coli, Haemophilus influenzae, Campylobacter jejuni, Rhodocyclus gelatinosus, Chromobacterium violaceum, Neisseria meningitidis, Salmonella minnesota.

In a first group of preferred glucosamine disaccharides according to the invention the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid, with this acyloxyacyl group at the 2'-position. In another preferred glucosamine disaccharide according to the invention the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{14}-fatty acid, and the acyloxyacyl group is preferably at the 2'-position.

In another preferred glucosamine disaccharide according to the invention the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid, with this acyloxyacyl group at the 2-position. In another preferred glucosamine disaccharide according to the invention the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid, with the acyloxyacyl group at both the 2-position and the 2'-position.

When the group Y comprises a chiral centre the invention encompasses all R- and S enantiomers, and any racemic mixture.

The other N-linked substituent may be an acyl group or also an acyloxyacyl group. According to a second group of disaccharides according to the invention the acyl group is a 3-hydroxy(C_4-C_{24})-fatty acid, preferably a

3-hydroxy(C_{10}-C_{18})-fatty acid. In the preferred disaccharides according to the invention the acyl group is a 3-hydroxyC_{14}-fatty acid, at the 2-position or at the 2'-position.

However, the N-linked substituent may also be an acyloxyacyl group defined hereinbefore, and comprising an N-linked 3-hydroxy(C_4-C_{24})-fatty acid-acyl which is ester-linked at the 3-hydroxy position with (C_1-C_{20})-carboxylic acid, preferably an N-linked 3-hydroxy(C_8-C_{18})-fatty acid-acyl ester-linked at the 3-hydroxy position with (C_{10}-C_{18})-fatty acid. More preferred is the disaccharide wherein R_2 is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid or C_{16}-fatty acid, and wherein R_2' is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid or C_{14}-fatty acid.

It is noted, that in the group Y the acyl groups and/or the acyl and alkyl group may be interlinked.

In this specification the term "fatty acid residue" means: a substantially hydrophobic chain of C_2-C_{30} atoms, which chain may be straight, branched, saturated, mono- or poly-unsaturated, having inserted one or more hetero atoms such as nitrogen, oxygen, sulphur, and which chain may be substituted with one or more substituents, such as hydroxyl, oxo, acyloxy, alkoxy, amino, nitro, cyano, halogeno, sulphydryl, provided that the biological activity is not substantially adversely affected. An example of a substituted fatty acid residue (comprising an amide-linked substituent) is disclosed by Onozuka, K. et al. in Int. J. Immunopharmac, Volume 15, pages 657-664 [1993]).

The substituent R_1 may be a (C_1-C_5)acyloxy group or a (C_1-C_5)alkyloxy group while R_4' may be a (C_1-C_5)acyl group or a (C_1-C_5)alkyl group, provided that the properties of the glucosamine disaccharides are not adversely affected. Furthermore, R_1 may be an hydroxyl group and R_4 may be hydrogen. Preferably, R_1 and R_4' may each be a phosphorus containing group. In particular such a group at the 1-position or 4'-position may influence the biological activity, such as a different stimulation of cytokines (see Takada, H., and Kotani, S., in Bacterial Endotoxic

Lipopolysaccharides, Morrison, D.C. and Ryan, J., CRC Press, Volume 1, pages 107-134 [1992], in particular page 123). The preferred disaccharides according to the invention comprise a dihydroxyphosphonoyloxy group at the 1-position and a phosphono group at the 4'-position, which group for the 1-position is preferably in the α-configuration.

The substituent R_1 may also be represented by the group X. The group X is generally negatively charged at physiological pH. The group X may be a carboxy (C_1-C_5)alkyloxy group. The group X may also be a dicarboxylic acid with the formula $-O-CH-[(CH_2)_mCOOH][(CH_2)_nCOOH]$, wherein m = 0-10 and n = 0-10, such as m and n = 0, m and n = 1; and m = 1 and n = 3. The dicarboxylic acid substituent at the 1-position wherein m and n = 1 is disclosed by Onozuka et al. (1993). Instead of a dicarboxylic acid the group X may be represented by a phosphono (C_1-C_5)-alkyl group, such as a phosphonomethyl group or a phosphonoethyl group.

The substituent group X may also have the form of a sulphate group or a hydroxysulphonyl (C_1-C_5)-alkyl group, such as a hydroxysulphonylmethyl group.

The substituents R_3 and R_3' may be a short alkyl or acyl group, which do not adversely affect the endotoxicity and/or biological activity of the glucosamine disaccharides according to the invention. Examples are a (C_1-C_3)alkyl group, and a (C_1-C_3)acyl group. Preferably, the substituents R_3 and R_3' are both hydrogen, that means that the 3-position and the 3'-position are not acylated.

The substituent R_4 at the 4-position may be a (C_1-C_3)alkyl group or a (C_1-C_3)acyl group of which the meaning has been defined hereinbefore. The 4-O-acylated disaccharide may be synthesized using the method disclosed by Kusumoto S., et al., ACS Symposium Series, Volume 231, pages 237-254, (1983). However, preferred is at the 4-position a hydroxyl group (R_4=H).

The substituent R_6' may be hydrogen, an hydroxyl group, a dihydroxyphosphonoyloxy group, a dihydroxysulphonyloxy group and their charged forms.

In order to improve the water-solubility of the glucosamine disaccharides according to the invention, the

substituent at the 6'-position may have a pronounced hydrophilic character imparted by the group Z. The group Z may be 3-deoxy-D-manno-2-octulosonic acid (KDO) or several KDO molecules, such as present in the inner core of natural polysaccharides directly adjacent to the lipid A component.

The group Z may also be the complete or partial polysaccharide chain, such as a side chain originating from natural lipopolysaccharide, or a core component originating from natural lipopolysaccharides.

The group Z may also be an amino-(C_1-C_8)alkyl-carboxyl group.

The water-solubility of the disaccharides according to the invention is on the one hand determined by the presence of charged group, the hydrophilic character of the substituent at the 6'-position. On the other hand, the water-solubility may also be improved when the glucosamine disaccharide is in the form of a salt, such as a salt comprising one or more alkali metal cations and/or ammonium ion forming a pair with for instance, dihydroxyphosphonoyloxy groups, carboxylate groups, phosphono groups, hydroxysulphonyloxy groups, and hydroxysulphonylalkyl groups when present.

It is noted that any alkyl and acyl chain or moiety may be straight, branched, saturated, mono- or poly-unsaturated, having inserted one or more hetero atoms such as nitrogen, oxygen, sulphur, and which chain may be substituted with one or more substituents, such as hydroxyl, oxo, acyloxy, alkoxy, amino, nitro, cyano, halogeno, sulphydryl, provided that the biological activity is not substantially adversely affected.

The glucosamine disaccharides according to the invention may be obtained from starting material comprising the lipid A moiety of lipopolysaccharides which are present in micro-organisms, such as Gram-negative bacteria. These lipopolysaccharides are for instance present in a surface structure comprising fraction of these micro-organisms and in lipopolysaccharides originating therefrom. Preferred Gram-negative bacteria used as a source for starting material are Escherichia coli and Haemophilus influenzae. However,

commercially available LPS or lipid A may be used as starting material.

The selective deacylation at the 3-position and at the 3'-position is carried out using an alkaline treatment. The conditions of the alkali treatment are chosen such that both glucosamines are 3-hydroxy deacylated. The alkaline treatment may be carried out using hydroxides, carbonates, and phosphates, such as sodium hydroxide or potassium carbonate. Illustrative organic alkaline agents are alkyl amines, such as diethylamine and triethylamine. The alkaline treatment is normally carried out in an aqueous or organic medium. The pH is typically within the range of 10-14, such as 11-13, under practical conditions the pH is for instance 12.2. The alkaline treatment is normally carried out at a temperature between ambient temperature and 70°C, such as 37°C. The time period depends on the type of starting material. Starting from micro-organisms the time period varies between 1 hour and 10 days, such as 8 hours and 5 days, but is normally within the range of 8-40 hours. Starting from lipopolysaccharides or lipid A the time period may be 0.2-10 hours, such as 1-5 hours. In practice the time period is about 1.5 to 3 hours.

When the starting material comprises at the 6'-position a core component that is to be removed, the starting material is to be subjected to an acid treatment for removing that core component. This acid treatment may be carried out before or after the afore-mentioned alkaline treatment. The acid treatment is carried out at a pH of 1-5, preferably in a pH range of 2.5-4.5, normally at a pH higher than 3 and lower than 4.5, such as 3.5. At pH 1 or below the glucosamine disaccharide is dephosphorylated, resulting in the monophosphorylated form. Acids that might be used are mineral and organic acids, such as hydrochloric acid and glacial acetic acid. The time period for the acid treatment is about 30 minutes to 5 hours, such as 1-2 hours. During the acid treatment the temperature is increased to about 70-100°C, such as 80-100°C, in practice 95°C. Subsequently the temperature is decreased to ambient temperature.

The glucosamine disaccharides according to the invention may also be obtained starting from the corresponding de-, mono-, or di-phosphorylated glucosamine dimer by attaching an acyloxyacyl group, acylaminoacyl group and/or an acylthioacyl group at both the 2-position and 2'-position.

Following partial deacylation of these glucosamine disaccharides according to the invention and separation of the products glucosamine disaccharides are obtained having the branched acyl group at the 2-position or at the 2'-position.

The glucosamine disaccharides according to the invention may be used in a pharmaceutical composition or medicament and used as an immunomodulating agent for inhibiting, stimulating or inducing the tolerisation of the production of nitric oxide reactive intermediates and cytokines, depending on the frequency of application and on the dosage, as anti-tumor agent, as for instance T-cell reactivation, as a vaccine component, as a competitor for endotoxin binding sites and as a modulator of interleukins. Due to the extremely low endotoxicity these disaccharides are almost or substantially free of side effects.

The disaccharides according to the present invention may be applied systemically or locally using intravenous injection, subcutaneous injection, intraperitoneal injection, intramuscular injection, and the like. The dosage will vary depending on the animal or human patient, age, body weight, symptoms or disease to be treated, the desired therapeutic effect, the administration route, term of treatment, and the like. Satisfactory effects will be obtained using a dosage of 0.001 to 500 mg/kg body weight administered in one or more daily doses or as a sustained release form.

The pharmaceutical composition may comprise a pharmaceutically acceptable carrier or diluent for, for instance, non-oral administration of aqueous or non-aqueous solutions, suspensions and emulsions. Aqueous solutions or suspensions may comprise distilled water or physiological saline. Non-aqueous solutions may include propylene glycol, polyethylene glycol, vegetable oils, such as olive oil,

alcohols. The composition may contain other additives, such as preservatives, wetting agents, emulsifying agents, dispersing agents and the like.

For a more complete understanding of the present invention reference is made to the following examples, which are provided herein for the purpose of illustration only, and are not intended to limit the scope of the present invention.

Example 1

Escherichia coli I-1147 (deposited at CNCM on October 3, 1991 under number I-1147) was cultured in a culture medium of which the composition is disclosed in table 1.

Table 1: composition of the culture medium (dissolved in water) for E. coli I-1147

Substance	amount/L
Inosine	0.200 g
Citric acid monohydrate	0.300 g
Glutamic acid	1.300 g
Ammonium chloride	1.050 g
Magnesium sulphate *$7H_2O$	1.110 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.360 g
Arginine	0.300 g
Uracil	0.100 g
Calcium chloride	0.017 g
Sodium chloride	2.000 g
Oligometals (stock 1000X conc)	1 ml
L-Leucine	10.0 g
L-Lysine.HCL	10.0 g
L-Serine	10.0 g
L-Methionine	10.0 g
L-Valine	10.0 g
L-Alanine	10.0 g
L-Asparagine	10.0 g
Glucose (Stock 500 g/l)	5 ml

Oligometals stock solution: 2.5 g $FeCl_2.4H_2O$, 0.25 g $CoCl_2.6H_2O$, 0.25 g $NaMoO_4.7H_2O$, 0.25 g $MnSO_4.4H_2O$, 0.25 g $ZnSO_4.7H_2O$, 0.25 g $NiSO_4.7H_2O$, 0.05 g H_3BO_4, 0.05 g $CuSO_4$, then add 1.0 L H_2O, mix and add 1.1 ml H_2SO_4 (85%).

The pH of the culture medium was adjusted using

5N NaOH, 5% ammonia or 25% HCL. Under aeration and stirring (500 rpm) Escherichia coli I-1147 was cultured at 37°C and a pH of 6.9.

Subsequently the content of the fermentor was inactivated using a thermal treatment (105°C for 2 minutes).

The inactivated content of the fermentor was ultrafiltrated (cut-off 1000 kD), and the retained bacteria were washed using an aqueous 0.6% NaCl solution. The washed bacterial suspension was concentrated by ultrafiltration. Biomass yield 764 g dry weight.

The biomass was diluted to 7.0 g/l and subjected to an alkali treatment by adding 345 ml 10.77 N NaOH and incubated at 37°C for 40 hours (pH 12.2).

The alkaline extract was subjected to a first ultrafiltration (cut-off 1000 kD), and to a second ultrafiltration of the permeate (10 kD). The retentate of the second ultrafiltration was subjected to an acid treatment.

The retentate was diluted with 7.0 l water and acidified using 370 ml glacial acetic acid (final pH 3.52). The mixture was heated to 95°C during 120 minutes while stirring. Subsequently the acid suspension was cooled to 25°C. The precipitate was separated by centrifugation (4000 x g during 50 minutes). The pellet was resuspended in water (3.7 l) and subjected to an extraction using propan-2-ol (4.3 l) and after 60 minutes at 25°C, 252 ml triethylamine was added (pH 9.0) and stirring was continued for 24 hours.

The supernatant was recovered by centrifugation (4000 x g, 25°C for 50 minutes) and the pellet was re-extracted two times using propan-2-ol 90%. The supernatants were combined and subjected to reversed-phase chromatography (Waters No. 10001, Preparative C_{18}, 125 Å).

Alternatively, the acid-treated extract was subjected to ultrafiltration and the retentate (> 1000 kD) was concentrated and dialyzed against 5 volumes water. The dialyzed retentate was diluted with 9 volumes propan-2-ol and adjusted to pH 9 with triethylamine (TEA). The extraction was carried out under stirring during 2 hours.

The supernatant is removed as described above and the precipitate is re-extracted with propan-2-ol. The

supernatants are combined and subjected to vacuum concentration (40°C, 12 Torr) and finally subjected to reversed-phase chromatography C_{18} Prep Sep Pak (Waters No. 10001).

Each of the two supernatants is diluted with two volumes of water and mixed with 5 mM tetrabutylammonium phosphate (TBAP) and applied to a column comprising 50 g reversed-phase C_{18} Prep Sep Pak (Waters No. 10001, Preparative C_{18}, 125 Å) preconditioned with 250 ml $CH_3CN:H_2O$ 1:1, (v/v) + 5 mM TBAP. The column was washed with 60% $CH_3CN:H_2O$ 1:1, (v/v) + 5 mM TBAP and 40% propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP. The disaccharides according to the invention eluted in a fraction at 30% $CH_3CN:H_2O$ 1:1, (v/v)+ 5 mM TBAP and 70% propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP.

The disaccharide fraction obtained in reversed phase chromatography is diluted with water 1:1 (v/v) + 25 mM TBAP and applied to a preparative HPLC column (Millipore-Waters Bondapak C18 300Å 15M, 300 mm x 47 mm ϕ). The disaccharide fraction according to the invention eluted at 67% propan-2-ol:H_2O 9:1, (v/v) + 25 mM TBAP and 33% $CH_3CN:H_2O$ 1:1, (v/v) + 25 mM TBAP. This fraction contained 55 mg of disaccharide A according to the invention.

Desalting of the disaccharide

Salt was eliminated from an aliquot of the disaccharide A fraction as follows. A Sep Pak Vac C_{18} Plus column (silica C_{18}, 0.6 ml, Waters No. 20515) was conditioned by successively injecting 5 ml of $CHCl_3$-CH_3OH 2:1, (v/v), 5 ml of CH_3CN and 5 ml of $CH_3CN:H_2O$ 1:1, (v/v). The sample was added to the column after dilution of the HPLC fraction with 3 volumes of H_2O, giving a total of 6 ml of diluted sample. The TBAP was then eliminated with 10 ml of $CH_3CN:H_2O$ 1:1, (v/v) + 10 ml mM HCl, followed by 10 ml of CH_3CN. The pure disaccharide A was then removed with 5 ml of $CHCl_3$-CH_3OH 2:1, (v/v).

The fraction was dried by evaporation under vacuum (12 Torr) at 35°C. The desalted disaccharide A was redissolved in H_2O:TEA 1000:1, (v/v) for biological and biochemical tests or in chloroform:methanol 2:1, (v/v) for FAB-MS.

Preparation of the sodium salt form

A column comprising 10 g reversed-phase C_{18} Prep Sep Pak (Waters No. 10001, Preparative C_{18}, 125 Å) was preconditioned successively with 50 ml of CH_3CN and 50 ml of $CH_3CN:H_2O$ 1:1, (v/v).

The sample (HPLC fraction) was added to the column after dilution with 1 volume of H_2O. After adsorption, the column was washed with 100 ml of $CH_3CN:H_2O$ 1:1, (v/v) + 5 mM TBAP. The disaccharide was then removed with 50 ml of propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP.

The resulting fraction was purified as follows. A column comprising 20 ml Q-Sepharose fast flow (Pharmacia 17-0510-01) was conditioned successively with 30 ml of NaOH 1M, washed with H_2O to neutralize, with 30 ml of HCl 1M and washed with H_2O to neutralize.

The sample was applied directly to the column. After adsorption the non adsorbed material was eliminated with 200 ml H_2O and 100 ml propan-2-ol:H_2O 9:1, (v/v). The disaccharide was eluted with 100 ml of NaCl 0.9%:isopropanol 1:1, (v/v).

The final purification was effected as follows. A column comprising 10 g reversed-phase C_{18} Prep Sep Pak was preconditioned successively with 50 ml of CH_3CN, 50 ml of $CHCl_3$-CH_3OH 2:1, (v/v), 50 ml of CH_3CN and 50 ml of 50% $CH_3CN:H_2O$ 1:1, (v/v). The sample was added to the column after dilution with 1 volume of H_2O. After adsorption, the column was washed successively with 200 ml of H_2O, 200 ml of propan-2-ol:H_2O 9:1, (v/v) and 50 ml of CH_3CN. The disaccharide was eluted with 50 ml $CHCl_3$-CH_3OH 2:1, (v/v). The fraction was dried by evaporation under vacuum (12 Torr) at 35°C.

The sodium salt was freely soluble in water (up to 100 mg/ml).

Example 2

Haemophilus influenzae (purchased from National Collection of Type Cultures (ATCC 9795)) was cultured in a culture medium of which the composition is disclosed in table 2.

Table 2: Composition of the main culture medium for Haemophilus influenzae

Substance	Amount/l
Sodium chloride	2 g
Sodium monohydrogen phosphate	2 g
Sodium acetate	0.5 g
Aneurine	0.003 g
Nicotinic acid	0.003 g
70% sodium lactate solution	2 ml
60% ammonium lactate solution	2 ml
Meat extract	7.5 g
Peptone	15 g
Soya peptone	1 g
Tryptone	3 g
Yeast extract	7.5 g
Glucose	3 g

The culture medium was supplemented with hemine (10 mg/l) and NADH (4 mg/l). The pH is adjusted to 7.0 ± 0.3 using 5 N NaOH or 25% HCl. After the beginning of the formation of a stationary phase culturing was interrupted and the content of the fermentor was inactivated by a thermal treatment (100°C for 100 seconds). The inactivated culture was subjected to centrifugation and the separated biomass was diluted with 0.6% aqueous NaCl solution (approximately 60 g/l). The alkaline treatment was carried out by adding 10 N NaOH to a final concentration of 0.2 N NaOH. The treatment is carried out at 37°C for 5 days under continuous stirring.

The alkaline-treated lysate was directly subjected to an acid treatment after acidification to pH 3.5 using glacial acetic acid. The mixture is heated to 95°C for 120 minutes and subsequently cooled to room temperature.

The precipitate was centrifuged (10,000 x g, 30 minutes at 4°C) and the supernatant discarded.

The precipitate was resuspended in $CH_3CN:H_2O$ 1:1, (v/v) and the pH was adjusted to pH 9 using TEA. After centrifugation (15,000 x g, 10 minutes) the supernatant was adjusted to 5 mM TBAP. The supernatant is applied to a Sep Pak Vak C_{18} column (10 g silica C_{18}, 35 ml, Waters No. 43345) conditioned using 50 ml $CH_3CN:H_2O$) 1:1, (v/v). The fraction

containing disaccharide B according to the invention was eluted with 50 ml propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP.

This fraction was concentrated by evaporation (35°C, 12 Torr) to about 2 ml. The fraction was centrifuged (15,000 x g, 5 minutes) and the supernatant was applied to a semi-preparative HPLC C_{18} column (Macherey-Nagel No. 715806, 250 mm x 10 mm ϕ, Nucleosil 300-7C18). The fraction containing disaccharide B according to the invention eluted in a fraction comprising 28% CH_3CN:H_2O 1:1, (v/v) + 25 mM TBAP and 72% propan-2-ol:H_2O 9:1, (v/v) + 25 mM TBAP.

Disaccharide B was desalted using a method similar to that of example 1.

Example 3

Lipopolysaccharide of Escherichia coli O111:B4 (Sigma, Product No. L3024) was subjected to an alkaline treatment in 0.2 M NaOH at 37°C during 1.5 hours. The solution was neutralized using 1 M phosphoric acid.

400 µl of the alkaline treated LPS solution was concentrated by ultrafiltration (Millipore Ultrafree-MC, UFC3 LGC 00, cut-off 10 kD).

The retentate (> 10 kD) was diluted in 400 µl H_2O and subjected to an acid treatment by adjusting to 0.2 M acetic acid using glacial acetic acid. The acidified solution was heated to 95°C for 120 minutes. After cooling to 25°C the precipitate was sedimented by centrifugation (15,000 x g, 10 minutes) and the supernatant was discarded. The precipitate was dissolved in 20 µl H_2O:TEA 1000:1, (v/v) and this solution was applied to an analytical HPLC C_{18} column (Supelco No. 58985, Supelcosil LC-18, 3µm, 150 mm x 4,6 mm ϕ). The disaccharide fraction according to the invention eluted in a fraction comprising 42% CH_3CN:H_2O 1:1, (v/v) + 5 mM TBAP and 58% propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP.

Example 4

A solution of 2 mg/ml lipid A of Escherichia coli F-583 (Sigma, Product No. L5399) was prepared in H_2O:TEA at 1000:1, (v/v), and this solution was subjected to an alkaline

treatment using 0.2 M NaOH at 37°C during 2.5 hours. The solution was neutralized using 1 M phosphoric acid.

The neutralized solution was applied to an analytic HPLC column (Supelco No. 58958, Supelcosil LC-18, 3 μm, 150 mm x 4.6 mm ϕ). The disaccharide according to the invention eluted at 42% $CH_3CN:H_2O$ 1:1, (v/v) + 5 mM TBAP and 58% propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP.

Example 5

2-Amino-2-deoxy-6-O-(2-amino-2-deoxy-4-O-phosphono-ß-D glucopyranosyl)-α-D-glucopyranosyl dihydrogenphosphate [Holst et al. Eur. J.Biochem. 214 (1993) 695-701] is treated in methanol with sodium methoxide (exactly 4.0 mol. equiv.) and then with (R)-3-dodecanoyloxytetradecanoic anhydride (2.2 mol. equiv.) [prepared by the reaction of (R)-3-dodecanoyloxytetradecanoic acid with DCC (0.5 mol. equiv.) in anhydrous dichloromethane, see Charon et al. J. Chem. Soc. Perkin Trans. I. (1984) 2291-2295]. After 12 hours at room temperature, water is added (2x volume of methanol) and the mixture is extracted with diethyl ether (to remove 3-dodecanoyloxytetradecanoic acid). The aqueous phase is concentrated and the crude disaccharide C according to the invention is subjected to reversed-phase HPLC. The product is dissolved in H_2O:TEA 1000:1, (v/v) and tetrabutylammonium phosphate [TBAP] added to a concentration of 5 mM. This solution is then applied to a preparative HPLC column (Millipore-Waters Bondapak C18 300Å 15M, 300 mm x 47 mm ϕ). Disaccharide C is eluted with a gradient $CH_3CN:H_2O$ 1:1 (v/v) + 25 mM TBAP (solvent A) and propan-2-ol:H_2O 9:1 (v/v) + 25 mM TBAP (solvent B) (50%A / 50%B to 0%A:100%B at 1%/min, flow 80 ml/min.). Desalting is achieved as follows. The HPLC fraction containing disaccharide C is diluted with water then applied to a C_{18}-Sep Pak Vac Plus column (Waters) [C18 reversed-phase silica gel preconditioned successively with $CHCl_3:CH_3OH$ 2:1 (v/v), CH_3CN, $CH_3CN:H_2O$ 1:1 (v/v)]. TBAP is eliminated by washing successively with $CH_3CN:H_2O$ 1:1 (v/v), 10 mM HCl and CH_3CN. Pure disaccharide C is eluted with $CHCl_3:CH_3OH$ 2:1 (v/v).

Example 6

The aqueous phase containing disaccharide C obtained in Example 5 (before purification) is treated with aqueous sodium hydroxide (exactly 1.0 mol. equiv.; concentration leading to an initial pH of 12.5); after 24 hours at room temperature the mixture is adjusted to pH 6.5 to 7 and applied to a preparative HPLC column (Millipore-Waters Bondapak C18 300Å 15M, 300 mm x 47 mm ϕ). The disaccharides A and D are eluted with a gradient $CH_3CN:H_2O$ 1:1 (v/v) + 25 mM TBAP (solvent A) and propan-2-ol:H_2O 9:1 (v/v) + 25 mM TBAP (solvent B) (75%A : 25%B to 0%A : 100%B at 1%/min, flow 80 ml/min). The HPLC fractions containing disaccharides A and D are desalted as described for disaccharide C in example 5.

Comparative example (not according to the invention)

A solution of 10 mg/ml lipid A from Escherichia coli F-583 (Sigma Product No. L5399) was prepared in H_2O:triethylamine at 1000:1, (v/v) and subsequently subjected to an alkaline treatment with 0.2 M NaOH at 37°C for 20 minutes. This time period was sufficient to only 3-O deacylate lipid A (Myers et al. in Cellular and Molecular Aspects of Endotoxin Reactions, pages 145-156 [1990], Elsevier Science Publishers).

The solution was neutralized with orthophosphoric acid. For biological assays it was diluted into 0.1% TEA/0.9% NaCl and used without further purification.

For FAB-MS a sample of this alkali-treated lipid A was purified by reversed-phase HPLC (Supelco No. 58985, Supelcosil LC18, 3 μm, 15 mm x 4.6 mm ϕ). A major peak eluting at 18% $CH_3CN:H_2O$ 1:1, (v/v) + 5 mM TBAP and 82% propan-2-ol:H_2O 9:1, (v/v) + 5 mM TBAP was desalted under conditions described in example 1. The FAB-MS analysis gave a molecular ion of 1570.1 mass units (calculated 1569.1 mass units).

Physico-chemical characteristics of disaccharides according to the invention

The disaccharides A and B obtained in examples 1 and 2 have been subjected to physicochemical characterizati...

Glucosamine was determined after acid hydrolysis (4 M HCL, 16 hours, 100°C, argon atmosphere) and derivatisation using phenyl isothiocyanate and subsequent quantitative analysis by HPLC (see Anumula, K.R. et al, Analytical Biochemistry, Volume 179, pages 113-122 [1991]).

Total fatty acids were determined after acid hydrolysis (4 M HCl, 4 hours, 100°C) by methylation using BF_3 in the presence of methanol and quantitative determination by gas chromatography (column OV-1, Hewlett Packard) (see Miller, L., Gas-Liquid Chromatography of Cellular Fatty Acids as a Bacterial Identification Aid, Gas Chromatography Application Note, pages 228-237 [1984]).

Ester-linked fatty acids were determined by gas chromatography after treatment using $NaOCH_3$ (see Rietschel, E.T. et al, European Journal of Biochemistry, Volume 28, pages 166-173 [1972]).

Phosphate was determined according to the method of Ames (see Ames, B.N., Methods in Enzymology, Volume 8, page 115-118 [1966]).

3-Deoxy-D-manno-2-octulosonic acid (KDO) was determined using the method of Karkhanis, Y.D. et al. (Analytical Biochemistry, Volume 58, pages 595-601 [1978]).

A solution of disaccharide A comprised 2.1 µmol/ml phosphate, 1.9 µmol/ml glucosamine, 1.0 µmol/ml $C_{12:0}$ fatty acid and 2.2 µmol/ml 3OH-$C_{14:0}$ fatty acid. Only the $C_{12:0}$ fatty acid was detected after release of ester-linked fatty acid residues, showing that the 3OH-$C_{14:0}$ fatty acid residues were amide linked. KDO was not detected (< 1 mol per 10 mol disaccharide A).

Accordingly, the disaccharide contains per mole, 2 moles of phosphate, 2 moles glucosamine, 2 moles 3OH-$C_{14:0}$ fatty acid and 1 mole $C_{12:0}$ fatty acid.

The fast atom bombardment mass spectroscopy (FAB-MS), negative mode of the sample in $CHCl_3$:CH_3OH 1:1, (v/v), concentration 1 mg/ml. A VG ZAB-2SE mass spectrometer set at V_{acc} 8 kV was used to generate a spectrum at 30 kV and an emission current of 1µA. The spectrometer is calibrated using cesium iodine. The FAB-MS spectrum is given in figure 1. Disaccharide A shows a molecular peak at 1133.55 mass units

(calculated mass 1133,3). Other peaks suggest a fragmentation of the product during analysis. The peak 1053.5 represents the loss of a phosphate group and 951.3 the loss of the C_{12} fatty acid.

The FAB-MS spectrum of disaccharide B is given in figure 2 and shows a molecular peak at 1161.8 mass units (calculated 1161.3). The peak at 1183.8 mass units represents the addition of sodium. The peak at 951.6 represents the loss of a C_{14} fatty acid. The peak at 973.6 mass units represents the fragment of the peak 951.6 with a sodium ion.

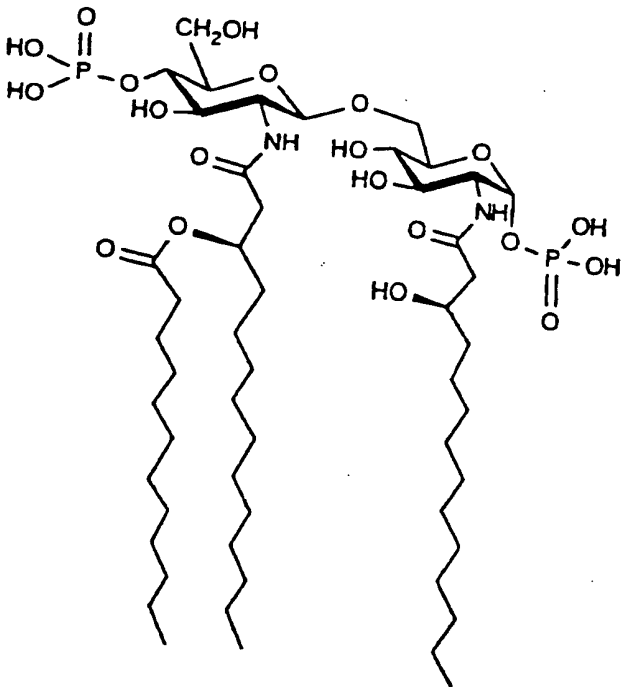
The ^{1}H-NMR-spectrum (Bruker 360 MHz) of disaccharide A (sodium salt in D_2O) is given in figure 3 and its ^{13}C-NMR-spectrum (Bruker 90 MHz) is given in figures 4 and 5 (expanded scale).

The structural formula of the following ß-D-glucosamine-(1-6)-α-D-glucosamine disaccharides:

* disaccharide A (2-Deoxy-6-O-[2-deoxy-2-[(R)-3-dodecanoyl-oxytetradecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate);
* disaccharide B (2-Deoxy-6-O-[2-deoxy-2-[(R)-3-tetradecanoyloxytetradecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate);
* disaccharide C (2-Deoxy-6-O-[2-deoxy-2-[(R)-3-dodecanoyloxytetradecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-dodecanoyloxytetradecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate);
* disaccharide D (2-Deoxy-6-O-[2-deoxy-2-[(R)-3-hydroxytetradecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-dodecanoyloxytetradecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate);

are disclosed hereafter.

A



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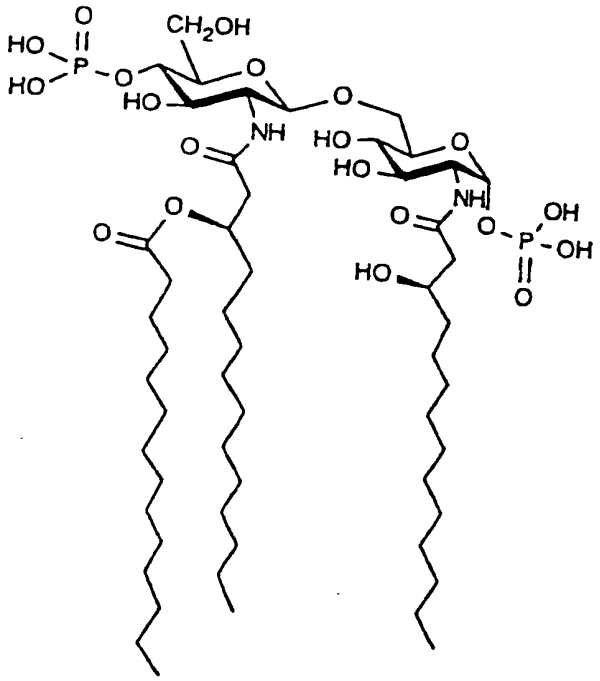

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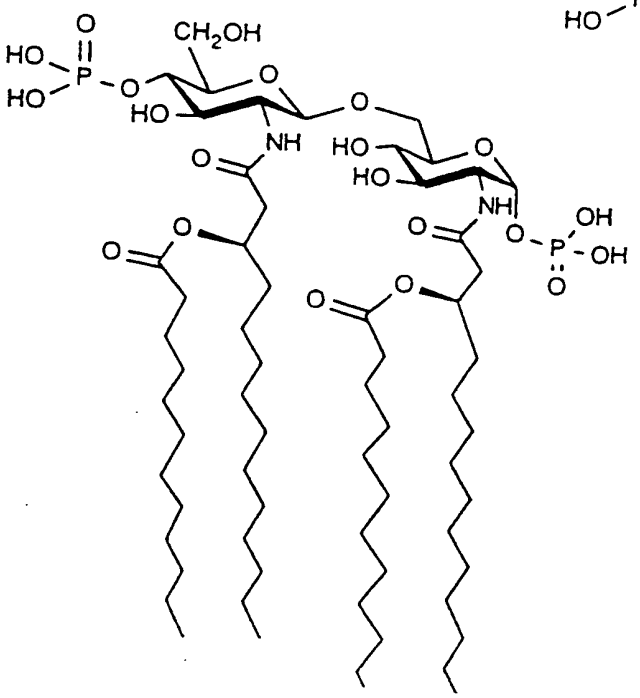

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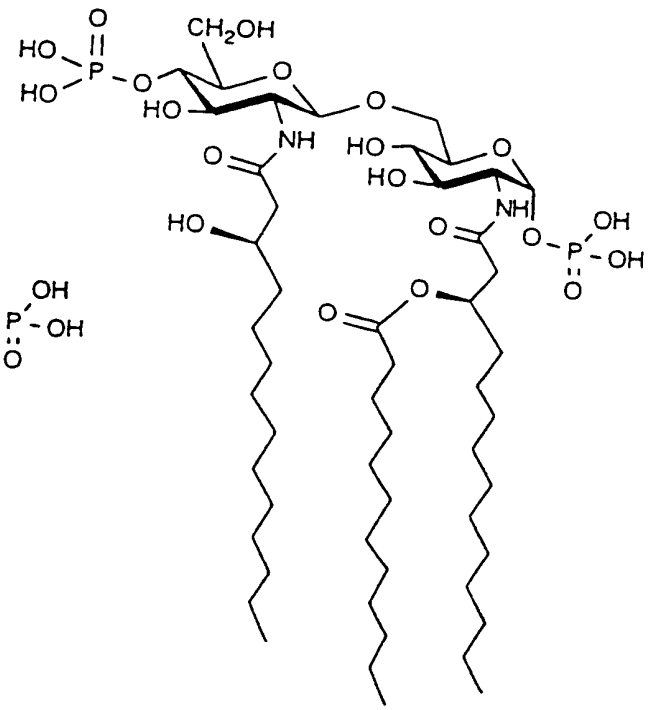


Endotoxicity and biological activity of disaccharides according to the invention

The endotoxicity and the biological activity of disaccharide A (example 1) and of disaccharide B (example 2) were determined and compared to that of lipopolysaccharide originating from Escherichia coli O111:B_4 (Sigma, Product No. L3024), lipid A (Sigma, Product No. L5399; used as a starting material in example 4), and 3-O-deacylated lipid A originating from lipid A of Escherichia coli F583 (prepared as in the comparative example), but without purification by HPLC.

1. Endotoxicity

The endotoxicity was determined in the Limulus amoebocyte lysate (LAL) test. This test is based on the observation that endotoxins induce coagulation of the hemolymph of Limulus polyphemus.

In the gelification test serial dilutions of the compound to be tested were mixed with LAL 1:1, (v/v) (Haemachem Inc., sensitivity LAL 0.06 endotoxin units/ml), and the mixture was incubated for one hour at 37°C. Then gel formation was monitored by measuring the optical density at 405 nm. The last dilution which formed a gel was determined by inverting the reaction microplate. The endotoxin activity in the samples was determined by comparison with dilutions of a lipopolysaccharide standard (1 endotoxin unit = 0.1 ng LPS).

The endotoxicity was also measured in the chromogenic test, in which the activation of a protease in LAL by LPS was measured using a chromogen (Ac-Ile-Glu-Ala-Arg-pNA; Bio Whittaker Kit No. 50-650U). The colour formation (liberation of pNA (p-nitroaniline)) was measured at 405 nm.

Samples were pre-incubated at 37°C during 10 minutes and subsequently chromogen-comprising LAL was added. The time required for reaching an optical density of 0.2 at 405 nm was measured. The endotoxin activity was calculated in comparison to a reference curve obtained for LPS standards.

The results are expressed in table 3 as: ng LPS per ng product.

Table 3: Endotoxic activity in LAL (ng/ng)

Type of test	E. coli LPS*)	Lipid A	3-O-deacylated lipid A	disaccharide A	disaccharide B	Monophosphoryl disaccharide A
Gelification	0.9±0.4 (n=6)	0.6±0.3 (n=3)	1.0±0.1 (n=2)	0.003±0.002 (n=12)	0.004±0.002 (n=7)	0.0031±0.0013 (n=10)
Chromogen	0.70 (n=1)	0.7±0.3 (n=3)	1.70±0.95 (n=3)	0.0014±0.0013 (n=9)	0.0008±0.0006 (n=7)	0.0016±0.0009 (n=10)

*) Endotoxic activity of LPS in LAL = 1 ng/ng, experimental data are within the range of 0.3-3 ng/ng.

Thus table 3 shows that disaccharides A and B according to the invention exhibit the lowest endotoxicity, in particular with regard to 3-O-deacylated lipid A according to US-A-4,912,094.

2. In vitro biological activity induced in macrophages of C57BL/6 mice

Bone marrow was collected from hip, femur and tibia of six week old male C57BL/6 mice. After homogenization of the marrow suspension in Dulbecco modified medium and centrifugation, the pellet was resuspended in Dulbecco modified medium and the cells were cultivated at a concentration of $4x10^5$ cells per ml in the same medium supplemented with 30% supernatant of L-929 fibroblasts (a common source for colony stimulating factor 1 [CSF-1]) and 20% horse serum.

After 7 days the mature macrophages were collected and resuspended in Dulbecco modified medium comprising 5% foetal calf serum to a concentration of $7x10^6$ cells per ml. This cell suspension was mixed 1:1, (v/v) with samples diluted in the same medium and used in the biological tests performed in microplates with 70,000 cells/well (incubation at 37°C for 22 hours, 100% humidity and 8% CO_2).

Nitric oxide (NO) production

Nitric oxide (NO) is produced by macrophages in response to bacterial infection and in particular LPS. NO seems to have cytostatic and cytotoxic properties. NO is extremely reactive and rapidly converted by oxidation into nitrite and nitrate.

The nitrite formation was determined using the Griess test (addition of 1:1, (v/v) N-(1-naphthyl)ethylene diamine hydrochloride [1 g/l in water] and p-aminobenzenesulphonamide [10 g/l in 5% H_3PO_4]). The nitrite concentration in supernatants of activated macrophages was calculated in comparison to $NaNO_2$ standards.

The results are disclosed in table 4.

Table 4: NO-production in the supernatants of macrophages stimulated by LPS and derived products

Product	Maximum activity *)	Minimum activity *)	Activity 50% *)	NO activity at 50% in nmol NO_2^-/ml**)	Number of analyses
LPS E. Coli	100	0.0004	0.01	7	n = 6
Lipid A	50	0.005	0.07	5	n = 7
3-O-deacylated lipid A	50	0.005	0.16	5	n = 3
Disaccharide A	50	0.016	0.16	5	n = 6
Disaccharide B	16	0.0005	0.05	5	n = 1
Monophosphoryl disaccharide A	50	5	8	4	n = 1

*) Product concentration expressed as µg/ml, corresponding to induced NO activity.

**) Concentration in NO_2^-/ml extrapolated from the series dilution curve.

LPS induces the highest NO production. Lipid A, 3-O-deacylated lipid A, disaccharide A and disaccharide B induce NO production of the same order.

Thus disaccharides A and B induce NO-production in macrophages as strongly as lipid A and 3-O-deacylated lipid A.

Production of interleukin-1α (IL-1α)

IL-1α is produced by a number of cells including macrophages when stimulated by LPS. Some reported IL-1α activities include the activation of T-cells, induction of Il-2 receptor expression and cytokine gene expression in T-cells, co-stimulation of B-cell-proliferation and Ig secretion, and augmentation of Il-2 and IFN-induced activation of NK-mediated cytotoxicity, induction of acute phase protein synthesis and fever induction.

The concentration of IL-1α in the supernatants of macrophages was measured by an ELISA-test (Kit GENZYME, Intertest-1α).

The results are summarized in table 5.

Table 5: Production of IL-1α in the supernatants of macrophages stimulated by LPS and derived products

Product	Highest concentration tested		Concentration 500 µg/ml	Minimum activity detected	
	concentration [µg/ml]	IL-1α [pg/ml]	IL-1α [pg/ml]	concentration [µg/ml]	IL-1α [pg/ml]
Blank = TEA 0.1%	500	<15*)	<15*)		<15*)
LPS E. coli	1600	295±129	170**)	1.56	18±4
Lipid A	500	53±35	53±35	50	17±8
3-O-deacylated lipid A	500	56±19	56±19	160	21±6
Disaccharide A	500	75±45	75±45	16	19±11

*) The limit of test detection is in the order of 15 pg/ml.

**) IL-1α concentration extrapolated from the series dilution curve.

It was not possible to determine the maximum production of IL-1α, because the production was still increasing at the highest concentration used.

The induction of IL-1α production at a concentration of 500 µg/ml is not significantly different for lipid A, 3-O-deacylated lipid A and disaccharide A. LPS induces IL-1α production more strongly.

The IL-1α production by disaccharide A is at least as strong as by lipid A and 3-O-deacylated lipid A.

Production of interleukin-6 (IL-6)

IL-6 is produced by activated monocytes or macrophages, T- and B-lymphocytes. IL-6 induces among others proliferation of certain types of cells, growth inhibition of certain melanoma cell lines, differentiation of B-lymphocytes and stimulation of IgG secretion, differentiation of cytotoxic T-cells, and a weak anti-viral activity.

IL-6 concentration in supernatants of macrophages was determined by an ELISA test (Kit ENDOGEN, EM-IL-6).

The results are summarized in table 6.

Table 6: Production of IL-6 in the supernatants of macrophages stimulated by LPS and derived products

Product	Maximum activity		Minimum activity		50% activity	
	concentration [µg/ml]	IL-6 [pg/ml]	concentration [µg/ml]	IL-6 [pg/ml]	concentration [µg/ml]	IL-6 [pg/ml]
Blank = TEA 0.1%	500	1150±80	0	710±240	---	---
LPS E. coli	25	13860±2750	0.006	2400±960	0.3	6950
Lipid A	160 to 0.016*)	3000 to 2200*)	0.016	2460±50	ND**)	ND**)
3-O-deacylated lipid A	160 to 0.016*)	2850 to 2200*)	0.016	2410±160	ND**)	ND**)
Disaccharide A	50	5700±2650	0.016	850±350	1	2850

*) The induced activity is relatively constant within the tested range and does not give a maximum.

**) ND = not determined.
The activity induced by the weakest concentration of tested product (0.016)
is still greater than the 50% activity.

The stimulation of IL-6 secretion by disaccharide A is significantly lower than for LPS. However, disaccharide A induces IL-6 production in macrophages more strongly than lipid A and 3-O-deacylated lipid A.

Production of tumor-necrosis-factor-alpha (TNF-α)

TNF-α is mainly produced by macrophages and monocytes stimulated by LPS. The activities induced by TNF-α are inter alia an anti-viral activity, cytolysis and cytostasis of certain cell types, growth of certain cellular lines, antigen expression such as major histocompatibility complexes class I and II, necrosis of methylcholanthrene-induced sarcoma,

and II, necrosis of methylcholanthrene-induced sarcoma, activation of polymorphonuclear leukocytes (PMN), osteoclast activation and bone resorption. TNF-α is also a principal mediator in toxic shock and sepsis.

The concentration of TNF-α in supernatants of macrophages was determined by an ELISA test (Kit GENZYME, Factor-test mTNF-α).

The results are listed in table 7.

Table 7: Production of TNF-α in the supernatants of macrophages by LPS and derived products

Product	Highest concentration tested		Concentration 500 µg/ml	Minimum activity detected	
	concentration [µg/ml]	TNF-α [pg/ml]	TNF-α [pg/ml]	concentration [µg/ml]	TNF α [pg/ml]
Blank = TEA 0.1%	500	138±9	138±9	0	<100*)
LPS E. coli	100 to 6.25	257 to 284	130***)	0.006	175±55
Lipid A	500	223±64	223±64	0.016	118±9
3-O-deacylated lipid A	500	311±72	311±72	0.016	155±6
Disaccharide A	500	530±139	530±139	0.16	159±43

*) The limit of test detection is in the order of 100 pg/ml.

**) TNF-α concentration is relatively constant between 100 and 6.25 µg/ml of LPS, and decreases for concentrations greater than 200 µg/ml.

***) TNF-α concentration is extrapolated from the series dilution curve.

It was not possible to determine the maximum production of TNF-α, because the production was still increasing at the highest concentration tested.

In contrast to the other tests, the TNF-α production induced by LPS was lower than for disaccharide A. Disaccharide A induced TNF-α equally or more strongly than lipid A or 3-O-deacylated lipid A.

Prostaglandin E2 (PGE2) production

PGE1 and PGE2 are the main metabolites of arachidonic acid synthesized by macrophages stimulated by LPS, TNF-α, or IL-1. PGE's exhibit immunomodulating activities on T- and B-lymphocytes. They seem to induce a stimulation of the Th2 and an inhibition of Th1 T-lymphocyte sub-populations and a switch in the isotype of immunoglobulines.

The PGE2 concentration in the supernatants of macrophages was measured by a RIA-test (Kit PAESEL + LOREI, 36-104-6001 Prostaglandin E2 3H-RIA Kit).

The results are summarized in table 8.

Table 8: Production of PGE2 in the supernatants of macrophages stimulated by LPS and derived products

Product	Highest concentration inducing an activity		Lowest concentration inducing an activity	
	concentration [µg/ml]	PGE2 [pg/ml]	concentration [µg/ml]	PGE2 [pg/ml]
Blank = TEA 0.1%	500	<80*)	---	<80*)
LPS E. coli	1600	1120±135	6.25	153±29
Lipid A	500	<80*)	---	<80*)
3-O-deacylated lipid A	500	240±25	500	240±25
Disaccharide A	500	540±65	16	80±41

*) The test detection limit is of the order of 80 pg/ml.

It was not possible to determine the maximum production of PGE2, because the production was still increasing at the highest concentration used. The stimulation of PGE2 production by disaccharide A is significantly lower than for LPS. However, disaccharide A was more active than lipid A and 3-O-deacylated lipid A. Lipid A did not induce PGE2 production and 3-O-deacylated lipid A only at the highest concentration used.

Conclusion

The disaccharides according to the invention are active in vitro and induce the production of NO, IL-1α, IL-6, TNF-α and PGE2. The disaccharides according to the invention are as active, or even more active, than lipid A and 3-O-deacylated lipid A, but exhibit a substantially reduced endotoxicity as determined by the LAL test. The lower activity of lipid A and 3-O-deacylated lipid A could be due to differences in purity. Disaccharides A and B are purified by HPLC, lipid A is a commercial biological preparation (Sigma L-5399) and 3-O-deacylated lipid A is prepared according to US-A-4,912,054 starting from the commercial preparation of lipid A. The only product with greater activity is the LPS which induced generally a higher response and required a lower concentration to induce a significant signal.

3. In vivo biological activity

The in vivo biological activity of disaccharide A was investigated for anti-tumor activity against peritoneal carcinomatosis induced in BDIX rats. Pro b cells obtained according to the method of Martin (Martin, F. et al, International Journal of Cancer, Volume 32, pages 623-627 [1983]) were injected intraperitoneally in rat (10^6 cells per rat). After 10 days numerous solid nodules appear in the mesenterium in the milky spots and progressively invade the peritoneal cavity (see Lagadec, P. et al, Invasion and Metastasis, Volume 7, pages 83-95 [1987]). Hemorrhagic ascitis appeared after 4-5 weeks and all rats died within 8-12 weeks.

Immunotherapy started 14 days after injection of the tumoral Pro b cells. The treatment consisted of intraperitoneal injections of disaccharide A; doses 0.1, 0.3 and 0.8 mg/kg bodyweight. Disaccharide A was dissolved in an aqueous solution of 0.9% NaCl and 0.1% triethylamine. The rats received five injections once every 3.5 days. A control group was injected with the aqueous solution. Both groups comprised 10 rats.

6 Weeks after the injection of the tumoral cells autopsy was carried out. The extent of peritoneal

carcinomatosis was evaluated blindly and the rats were classified in the order of increasing carcinomatosis.

The classification of the nodule size is as follows:

class 0: no tumor nodules visible;
class 1: a few nodules of size less than 0.2 cm;
class 2: nodules too many to be counted size up to 0.5 cm;
class 3: tumors in size up to 1 cm;
class 4: tumoral cavity entirely invaded by tumors, size a few cm.

The results are reviewed in table 9.

Table 9: In vivo anti-tumoral activity of disaccharide A in peritoneal carcinomatosis

Disaccharide A (dose)	Number of rats with carcinomatoses of class:					Effect of product	Ascitis volume [ml/rat]		Effect of product
	0	1	2	3	4	(1)	Limits	Average	(2)
0 mg/kg	0	1	1	2	6		0-64	40±24	
0.1 mg/kg	0	2	0	4	4	NS	0-18	7±7	p<0.001
0.3 mg/kg	2	3	2	2	1	p<0.01	0-20	2±6	p<0.001
0.8 mg/kg	1	6	1	1	1	p<0.01	0-2	0±1	p<0.001

The statistical significance of the anti-tumoral activity was calculated by the Kruskal-Wallis test (1) or (2) using variance analysis.

Obviously, disaccharide A possesses a dose-dependent anti-tumoral effect.

4. Acute toxicity

Disaccharide A was injected into the caudal vein of male and female NMRI mice (age 6-7 weeks). A dose up of 100 mg/kg bodyweight did not induce any death.

Example 7

The starting material is lipopolysaccharide from Pseudomonas aeruginosa (Sigma, Product No. L7018). The structure of lipid A is already known (see Kulshin et al. in Eur. J. Biochem. 198 (1991) 697-704). In contrast to lipid A from E. coli, the predominant species contain acyloxyacyl

residues at both amino groups of the diglucosamine diphosphate backbone. In addition there is a 3-hydroxy-decanoic acid at position 3', but the same fatty-acyl residue is only present at position 3 in a minor fraction.

Removal of this fatty-acyl residue at position 3' would lead to an analogue of disaccharide C. Further hydrolysis would lead to loss of the esterified fatty-acyl residue at the acyloxyacyl group at either the position 2 or 2'. These structures are analogues to the disaccharides C and D.

Lipopolysaccharide from Ps. aeruginosa (Sigma L7018) was dissolved in 0.1 M sodium acatate pH 4.0 at 5 mg/ml and heated for 120 min. at 100°C. After cooling, 0.5 volumes of propan-2-ol were added followed by tetrabutylammonium phosphate (TBAP) to 25 mM final concentration. Triethylamine (TEA) was added to pH 9.0 (approx., pH papers). The mixture was applied to a C18 Sep-Pak (Waters) with recycling (10 passages). The Sep-Pak was washed with 10 ml 5mM TBAP in acetonitrile:H_2O 1:1 (v/v) followed by 10 ml acetonitrile. The adsobed substances were eluted with 4 ml chloroform:methanol 2:1 (v/v).

The two major peaks, PsA1 and PsA2 (figure 6) were purified by HPLC and the fatty acids analysed. The fatty acid composition corresponds to the molecules described by Kulshin et al. (table below). PsA1 is less hydrophobic than PsA2 as it elutes from the column with a lower retention time (R_t). This corresponds to the molecule with two 2OH-C12:0 acid residues. PsA2 has both 2OH-C12:0 and C12:0.

Peak	Fatty acid identified
PsA1	3OH-C10:0
	2OH-C12:0
	3OH-C12:0
PsA2	3OH-C10:0
	C12:0
	2OH-C12:0
	3OH-C12:0

The solvent was removed by rotary evaporation and the residue redissolved in 0.2% TEA in water.

Sodium hydroxide was added to the solution of Ps. aeruginosa lipid A to a concentration of 0.2 M and the solution was incubated at room temperature for 60 min. The solution was then neutralized with (8.5%) orthophosphoric acid. It was then applied to a reversed-phase HPLC system (HP1050 with a Supelco LC18, 3 μm reversed-phase column, with precolumn) equilibrated in 75% solvent A (5mM TBAP in acetronile water 1:1 (v/v) 25% solvent B (5mM TBAP in propan-2-ol:water 9:1 (v/v)) and eluted with a gradient of 2% solvent B/min to 100% B. Peaks were detected by absorption at 210 nm. The major peaks were collected (see figure 7). These were diluted with 2 volumes water and applied to C18 Sep-Pak cartridges equilibrated in solvent A. The Sep-Pak's were washed with 10 ml 0.45% sodium chloride in propan-2-ol:H_2O 1:3 (v/v), 10 ml water and 10 ml acetonitrile. The adsorbed substances were eluted with 4 ml chloroform:methanol 2:1 (v/v) and the solvents removed under nitrogen. The fractions were redissolved in 100 μl water.

The fatty-acyl content of the fractions was analysed by gas chromatography after hydrolysis in 4 M HCl, 100°C, 4h. The released fatty acids were converted to the methyl esters according to Miller (Miller, L. Gas Chromatography application note 228-37 [Hewlett Packard]) and analysed on a Hewlett-Packard 5890 gas chromatogram with a fused silica column (Supelco 2-4026) with reference to standard fatty acid methyl esters (Supelco).

After hydrolysis of the lipid A extract, many peaks are observed on reversed-phase HPLC. The main peaks (PsAOH1, 2, 4 and 6) were collected from the HPLC. The fatty-acids identified in each fraction are shown below.

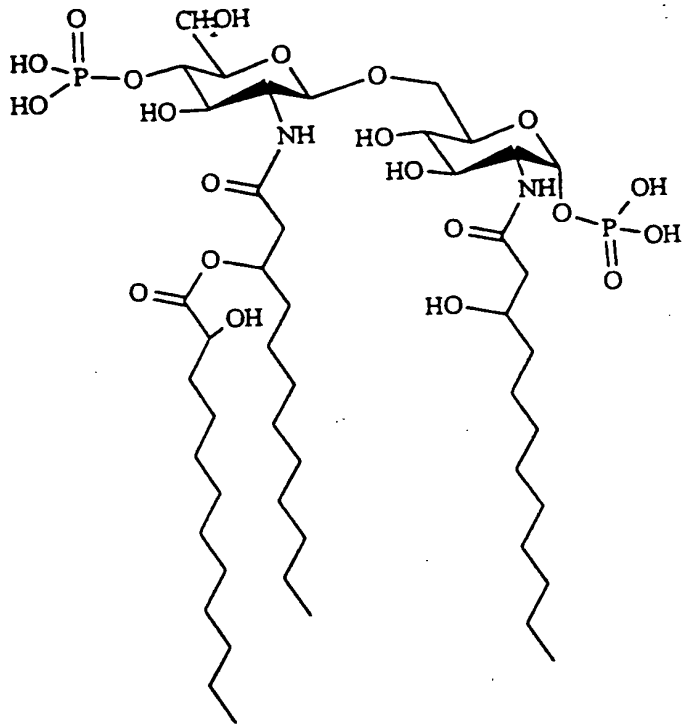
Peak	Fatty acid identified
PsAOH1	3OH-C12:0
	2OH-C12:0
PsAOH2	3OH-C12:0
	2OH-C12:0
PsAOH4	3OH-C12:0
	C12:0
PsAOH6	3OH-C12:0
	2OH-C12:0
	C12:0

The structural formula of these disaccharides are as follows:

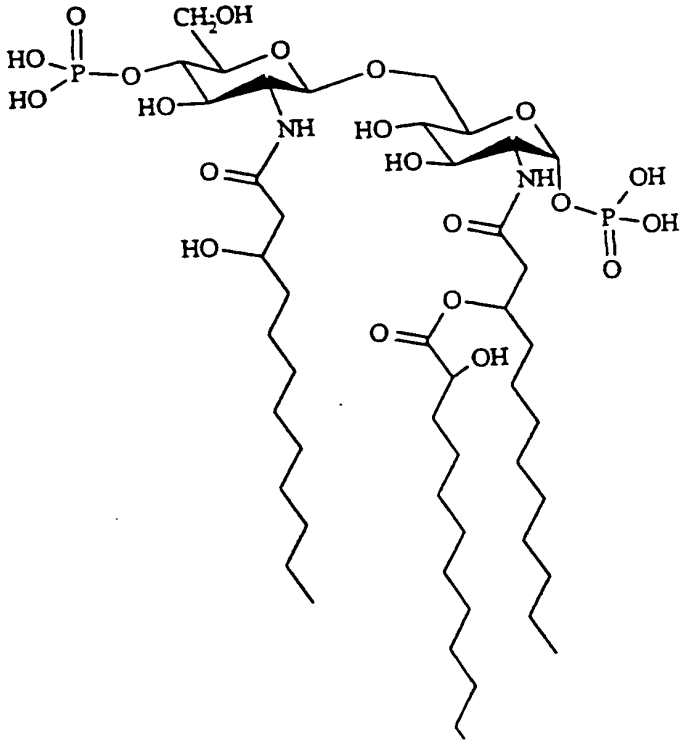
* PsAOH1: 2-Deoxy-6-O-[2-deoxy-2-[(R)-3-[(S)-2-hydroxydodecanoyloxy]-dodecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-hydroxydodecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate;
* PsAOH2: 2-Deoxy-6-O-[2-deoxy-2-[(R)-3-hydroxy-dodecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)--3-[(S)-2-hydroxydodecanoyloxy]-dodecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate;
* PsAOH4: 2-Deoxy-6-O-[2-deoxy-2-[(R)-3-dodecanoyloxydodecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-hydroxydodecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate;
* PsAOH6: 2-Deoxy-6-O-[2-deoxy-2-[(R)-3-dodecanoyloxydodecanoylamino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(R)-3-[(S)-2-hydroxydodecanoyloxy]-dodecanoylamino]-α-D-glucopyranosyl dihydrogenphosphate;

are disclosed hereafter.

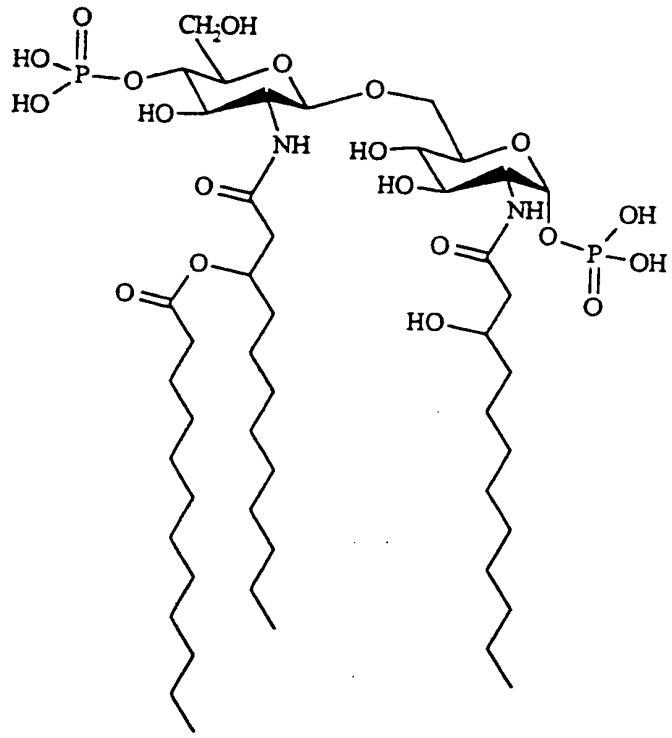
disaccharide E (PsAOH1)



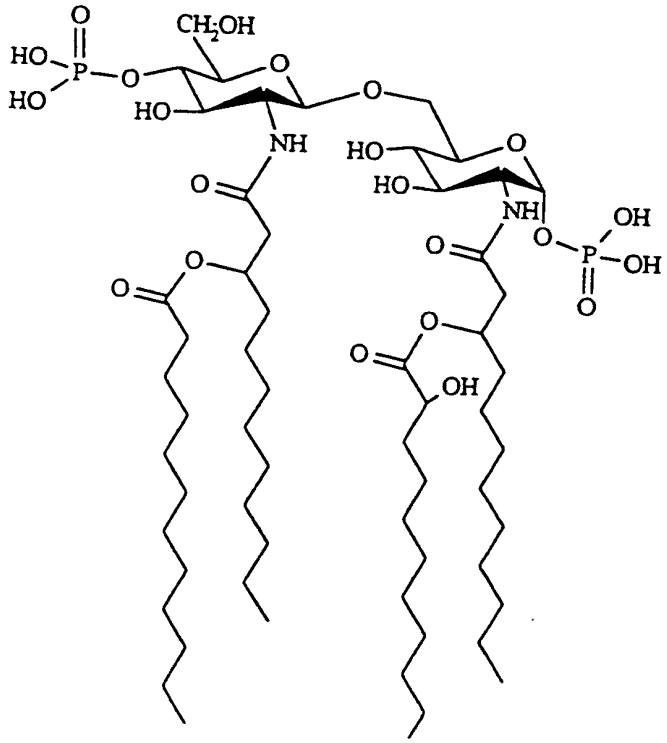
disaccharide F (PsAOH2)



disaccharide G (PsAHO4)



disaccharide H (PsAOH6)



The fractions were also analysed by electro-spray mass spectrometry (ES-MS) in the negative mode. A VG Biotech BIO-Q instrument was used with a triple quadrupole analyser. 2 to 4 µl of each sample was diluted into 10 µl acetonitrile:water:25% ammonia solution, 50:50:1 (v/v). 10 µl were then injected directly into the source of the mass spectrometer. Acetonitrile:water:25% ammonia solution, 50:50:1 (v/v) at 7 µl/min was used as eluant. For analysis of the fragmentation of the principle ions, parent ions from the first quadrupole were subjected to collision activated decomposition in the second quadrupole using argon as the collision gas. Daughter ions were detected in the third quadrupole.

The mass caclulated for each of the peaks and the mass observed by ES-MS are given below.

Peak	Calculated mass	Observed mass
PsAOH1	1093.5	1093.4
PsAOH2	1093.5	1093.1
PsAOH4	1077.5	1077.0
PsAOH6	1275.8	1275.7

There is a very good correspondence in each case.

In the case of PsAOH1 and PsAOH2 the masses are identical. This indicates that they represent two isoforms of the molecule, Lipid A's are known to fragment under certain analytical conditions in mass spectrometry (Kulshin, 1991; and Cotter et al., Biomed. Encl. Mass Spectrom. 14 (1987), 591-598). Ions are produced which represent the non-reducing half of the molecule with an addition of 102 mass units. Thus with two MS in tandem the principle ion in the first MS can be fragmented and the "daughter" ions detected in the second MS. This eliminates the possibility that the secondary ions observed are contaminants; they must come from the original ion by fragmentation. The mass of the daughter ions expected

for each fraction and the masses observed on MS-MS are shown hereafter.

Peak	Calculated mass of fragment	Observed mass of fragment
PsAOH1	558.5 or 756.8	756.1
PsAOH2	558.5 or 756.8	557.7
PsAOH4	558.5 or 740.8	739.9
PsAOH6	558.5 or 740.8	740.1

These observations clearly identify the structures of the disaccharides E, F, G, and H.

For internal comparison disaccharide A was also analysed by ES-MS. The calculated mass was 768.9 and the observed mass of fragment 768.

The biological activity of the fractions was tested by the stimulation of nitrite production in murine peritoneal macrophages as described hereinbefore.

The quantity of each analogue in the stock solution was determined from the absorption on HPLC with reference to that of disaccharide A. The fractions show activities of the same order as disaccharide A (figure 8). Disaccharide H, which has two acyloxyacyl groups and no other fatty-acyl residues, is the most active. The position of the acyloxyacyl, 2 versus 2', has only a minor influence on the activity.

In order to eliminate the possibility that activity was due to contamination of the samples by other substances such as LPS, disaccharides E, F, G and H were repurified on reversed-phase HPLC and the regions of the HPLC baseline just before and just after the peak were also collected and treated in the same way as the fractions containing the peaks of material. The activity of the peak fractions and the baseline fractions was tested. The peaks containing disaccharide E, F, G and H showed similar activity to that seen in the first assay. The blank samples, representing the regions of the HPLC profile just before and after the peak of

lipid A analogue, were inactive. The stimulation of nitrite production is thus specifically associated with the lipid A analogues.

The endotoxicity of the disaccharides E, F and G was determined using the chromogenic LAL test (see infra). However, instead of using 1 mg/ml bovine serum albumin, 0.1 mg/ml has been used. The results (n = 4 or 6) were obtained in two series of experiments and are shown hereafter.

Sample	Endotoxic activity in LAL (mg/mg)	
E.coli LPS	0.58	± 0.14
E.coli lipid A	0.32	± 0.06
disaccharide E	0.000005	± 0.000002
disaccharide F	0.000025	± 0.000026
disaccharide G	0.00006	± 0.00003
disaccharide A	0.000003	± 0.000002

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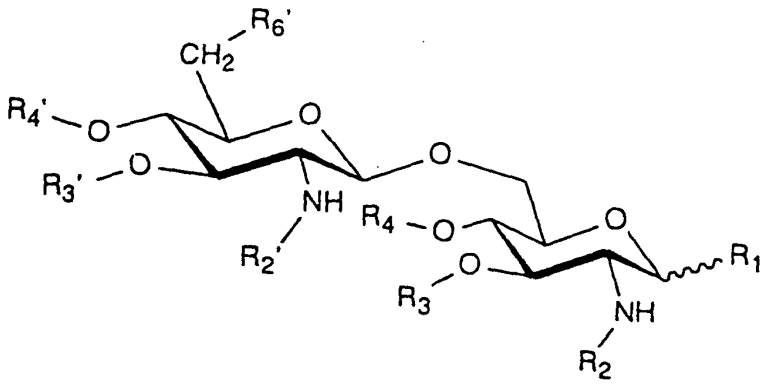
CLAIMS

1. ß(1→6) glucosamine disaccharide having the general formula



wherein

R_1 is a hydroxyl group,
a dihydroxyphosphonoyloxy group or its charged forms,
a (C_1-C_5)acyloxy group,
a (C_1-C_5)alkyloxy group, or
a group X;

R_2 and R_2' are each an acyl group or a group Y with the proviso that at least R_2 or R_2' is the group Y;

R_3 and R_3' are each hydrogen,
a (C_1-C_3)alkyl group, or
a (C_1-C_3)acyl group;

R_4 is hydrogen,
a (C_1-C_3)alkyl group, or
a (C_1-C_3)acyl group;

R_4' is hydrogen,
a (C_1-C_5)acyl group,
a (C_1-C_5)alkyl group, or
a dimethoxyphosphonoyl group, or

a phosphono group or its charged forms; and

R_6' is hydrogen,
a hydroxyl group,
a dihydroxyphosphonoyloxy group,
a hydroxysulphonyloxy group, their charged forms,
or a group Z;

wherein the group X is selected from the group comprising
a carboxy (C_1-C_5)alkyloxy group;
an $-O-CH-[(CH_2)_mCOOH][(CH_2)_nCOOH]$ group,
wherein m = 0-5 and
n = 0-5;
a phosphono(C_1-C_5)alkyl group;
a dimethoxyphosphonoyloxy group;
a hydroxysulphonyloxy group;
a hydroxysulphonyl(C_1-C_5)alkyl group; and
charged forms of the group X;

wherein the group Y is selected from the group comprising
an acyloxyacyl group,
an acylaminoacyl group,
an acylthioacyl group,
a (C_1-C_{24})alkyloxyacyl group,
a (C_1-C_{24})alkylaminoacyl group,
a (C_1-C_{24})alkylthioacyl group; and

wherein the group Z is selected from the group comprising
a (C_1-C_{24})alkyloxy group;
a (C_1-C_{24})acyloxy group;
3-deoxy-D-manno-2-octulosonic acid (KDO);
$(KDO)_n$, wherein n = 1-10;
a polysaccharide side chain, such as a side chain originating from natural lipopolysaccharide;
a core component, such as a component originating from natural lipopolysaccharide; and
amino-(C_1-C_8)alkyl-carboxyl group;

and its salts.

2. Disaccharide according to claim 1, wherein the group Y comprises a 3-acyloxyacyl group, a 3-acylaminoacyl group, and a 3-acylthioacyl group.

3. Disaccharide according to claim 1 or 2, wherein the group Y is an acyloxyacyl group.

4. Disaccharide according to claims 1-3, wherein the acyl group is a fatty acid residue, a 3-hydroxy fatty acid residue, a 3-oxo fatty acid residue.

5. Disaccharide according to claims 1-4, wherein the acyloxyacyl group, the acylaminoacyl group and the acylthioacyl group forming the group Y, comprise acyl moieties selected from the group comprising a fatty acid residue, a 3-hydroxy fatty acid residue, a 3-oxo fatty acid residue.

6. Disaccharide according to claims 3-5, wherein the group Y is an acyloxyacyl group which is an N-linked 3-hydroxy(C_4-C_{24})-acyl, preferably (C_8-C_{18})-fatty acid-acyl ester-linked at the 3-hydroxy position with a (C_1-C_{20})-acyl, preferably (C_{10}-C_{18})-fatty acid-acyl.

7. Disaccharide according to claim 6, wherein the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid.

8. Disaccharide according to claim 6, wherein the acyloxyacyl group is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{14}-fatty acid.

9. Disaccharide according to claims 1-8, wherein R_2'is the group Y.

10. Disaccharide according to claims 1-8, wherein R_2 is the group Y.

11. Disaccharide according to claims 1-10, wherein the 3-hydroxy fatty acid residue is a 3-hydroxy(C_4-C_{24})-, preferably 3-hydroxy(C_{10}-C_{18})-fatty acid.

12. Disaccharide according to claim 11, wherein the 3-hydroxy fatty acid residue is 3-hydroxyC_{14}-fatty acid.

13. Disaccharide according to claim 11 or 12, wherein R_2 is the 3-hydroxy fatty acid residue.

14. Disaccharide according to claim 11 or 12, wherein R_2' is the 3-hydroxy fatty acid residue.

15. Disaccharide according to claims 1-10, wherein R_2 and R_2' are both a group Y.

16. Disaccharide according to claim 15, wherein R_2 and R_2' are both a acyloxyacyl group comprising an N-linked 3-hydroxy(C_4-C_{24})-acyl, preferably (C_8-C_{18})-fatty acid-acyl ester-linked at the 3-hydroxyl position with a (C_1-C_{20})-acyl, preferably (C_{10}-C_{18})-fatty acid-acyl.

17. Disaccharide according to claim 16, wherein R_2 is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{16}-fatty acid, and wherein R_2' is the N-linked 3-hydroxyC_{14}-fatty acid-acyl ester-linked at the 3-hydroxy position with the C_{12}-fatty acid.

18. Disaccharide according to claims 1-17, wherein R_1 is a dihydroxyphosphonoyloxy group.

19. Disaccharide according to claims 1-18, wherein R_4 is hydrogen.

20. Disaccharide according to claims 1-19, wherein R_1 is in the α configuration.

21. Disaccharide according to claims 1-20, wherein R_3 is hydrogen.

22. Disaccharide according to claims 1-21, wherein R_3' is hydrogen.

23. Disaccharide according to claims 1-22, wherein R_6' is an hydroxyl group.

24. Disaccharide according to claims 1-23, wherein R_4' is a phosphono group.

25. Disaccharide according to claims 1-24, wherein the disaccharide is in the salt form comprising one or more cations.

26. Disaccharide according to claim 25, wherein the cations are alkali metal ions.

27. Method for preparing a disaccharide according to claims 1-26, comprising the steps of:

i) providing a starting material comprising lipid A moiety of lipopolysaccharide-comprising micro-organisms; and

ii) subjecting the starting material to an alkaline treatment such that lipid A moiety is O-deacylated at the 3-position and at the 3'-position.

28. Method according to claim 27, wherein the starting material is selected from the group comprising lipopolysaccharide-comprising micro-organisms, Gram-negative bacteria, a surface structure comprising fraction of these micro-organisms and Gram-negative bacteria, or a lipopolysaccharide of these micro-organisms and Gram-negative bacteria.

29. Method according to claim 27 or 28, wherein the starting material is lipid A of Gram-negative bacteria.

30. Method according to claims 27-29, wherein the alkaline treatment is preceded or followed by an acid treatment for removing the core moiety and the polysaccharide side chain.

31. Pharmaceutical composition comprising as an active ingredient a disaccharide according to claims 1-26, and/or a

disaccharide obtained in claims 27-30, and a pharmaceutically acceptable carrier or diluent.

32. Disaccharide according to claims 1-26 and/or obtained in claims 27-30 for use as an immunomodulating agent, and/or anti-tumor agent.

33. Disaccharide according to claims 1-26 and/or obtained in claims 27-30 for use as a vaccine component.

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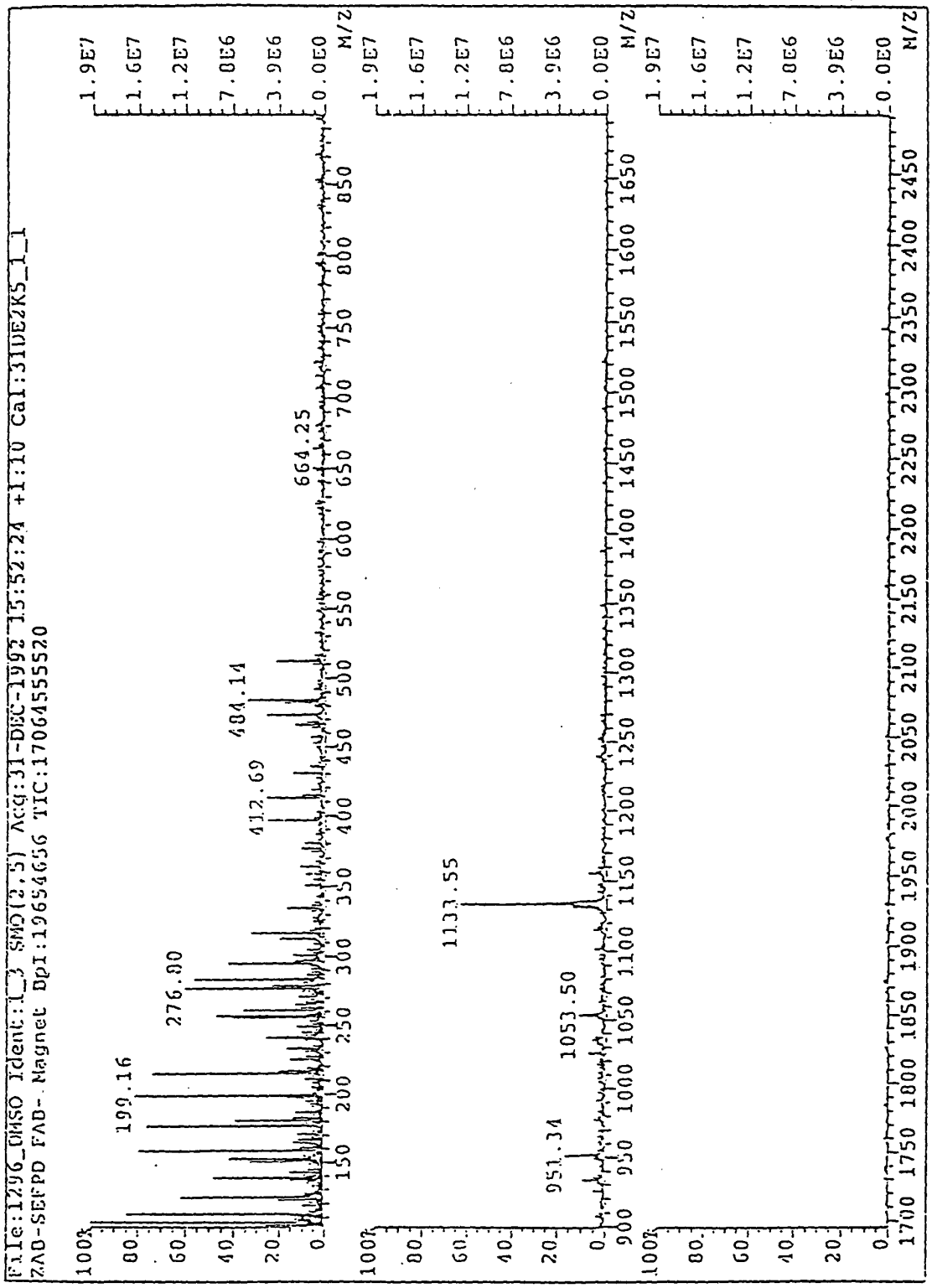

Fig. 1

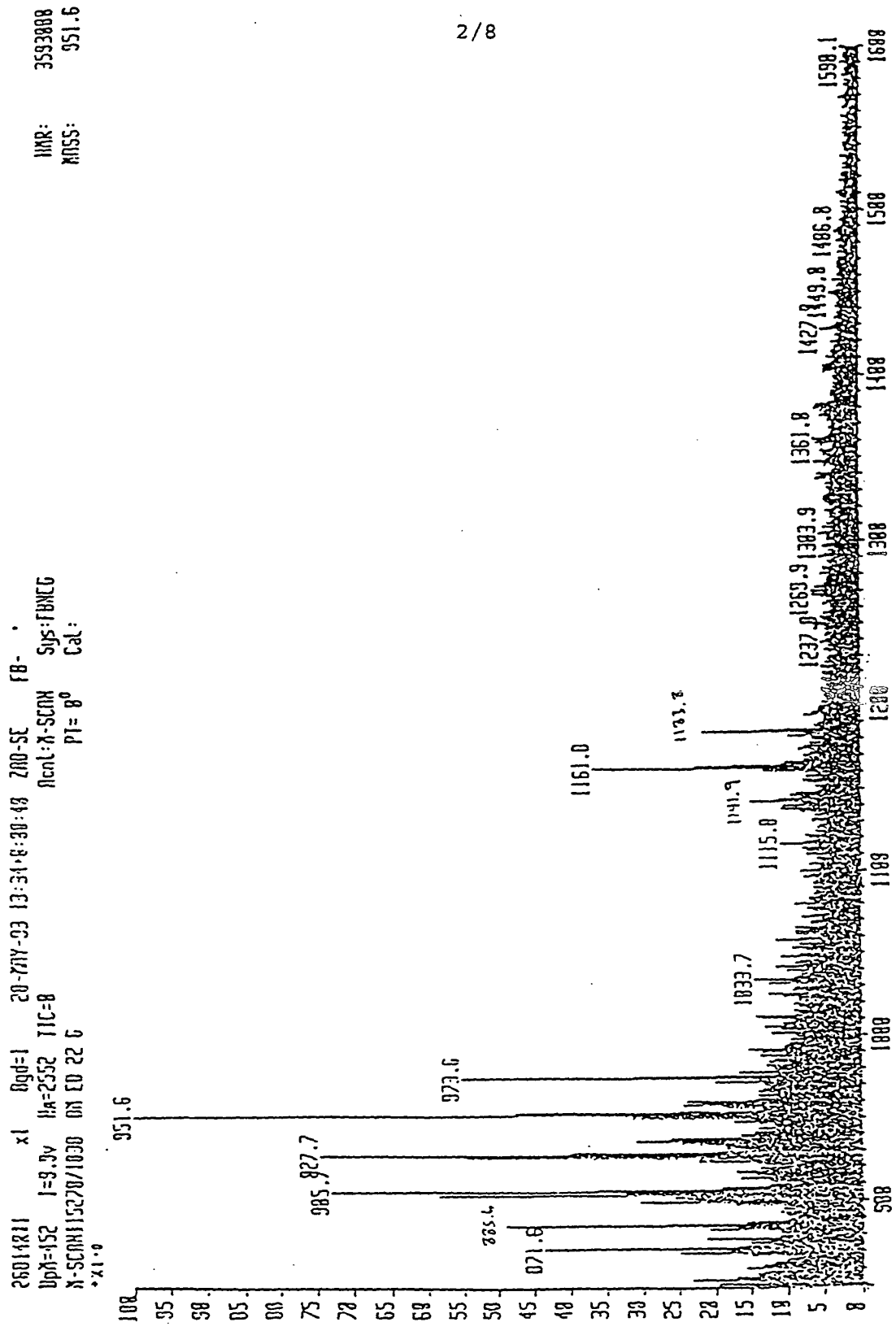

Fig. 2

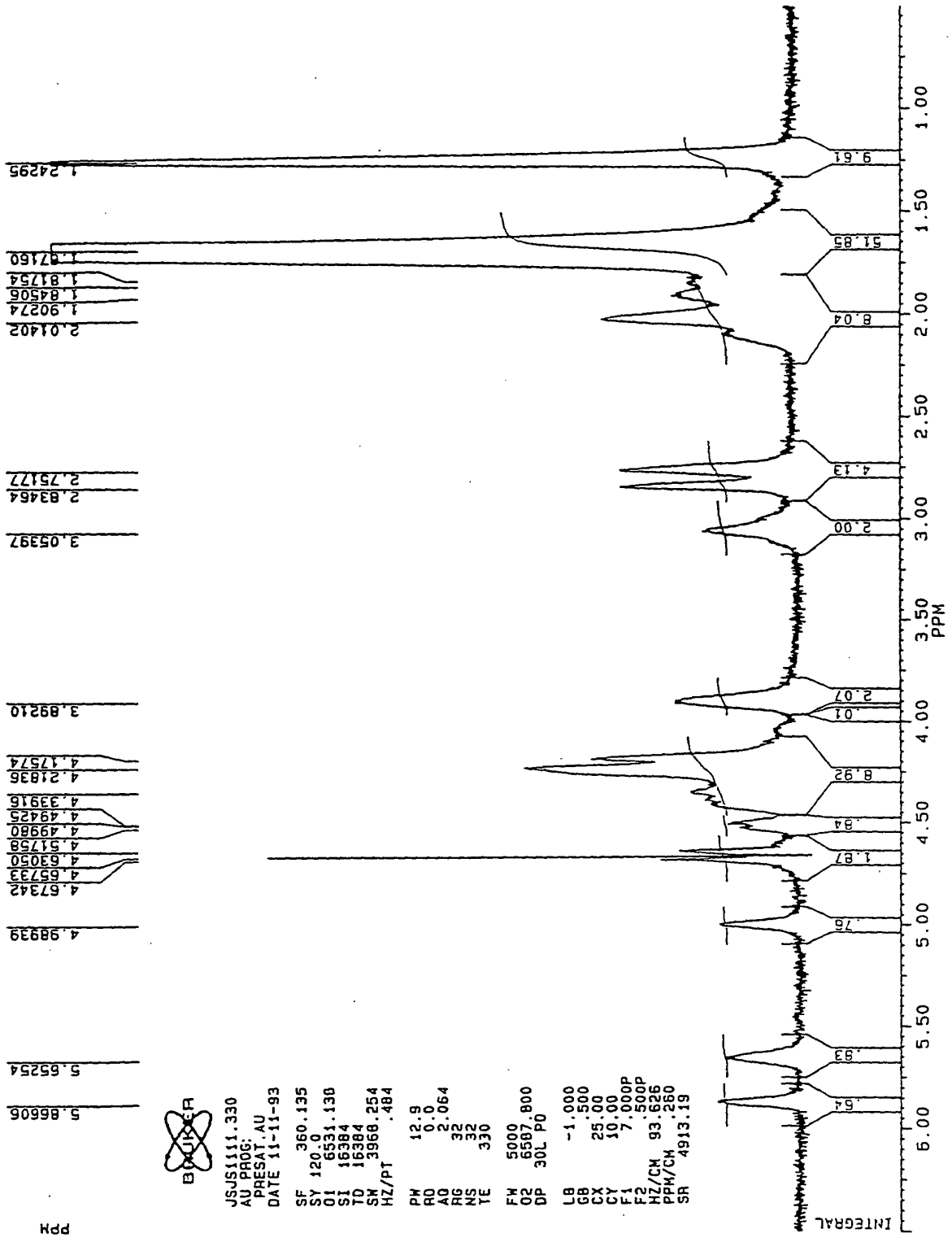

Fig. 3

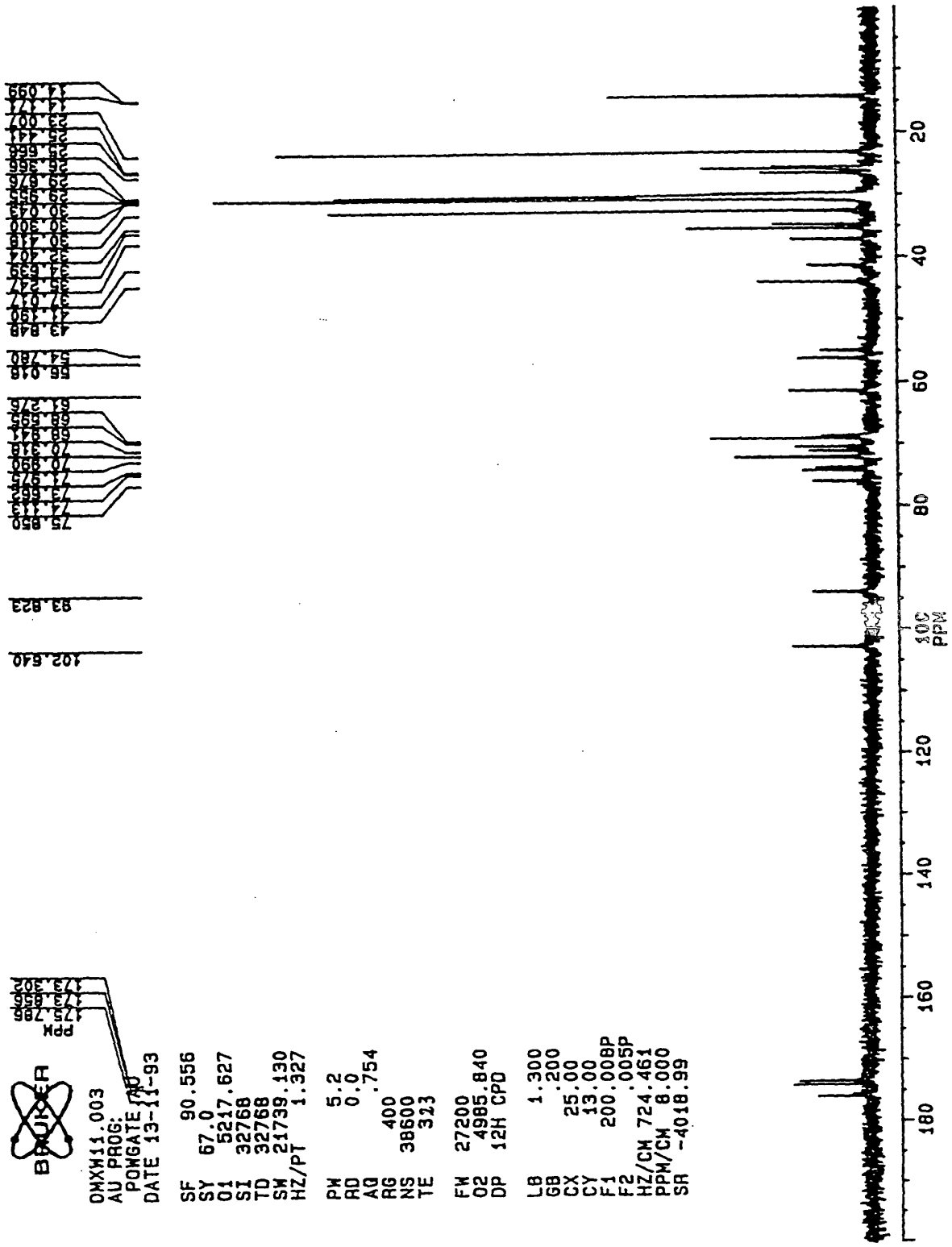

Fig. 4

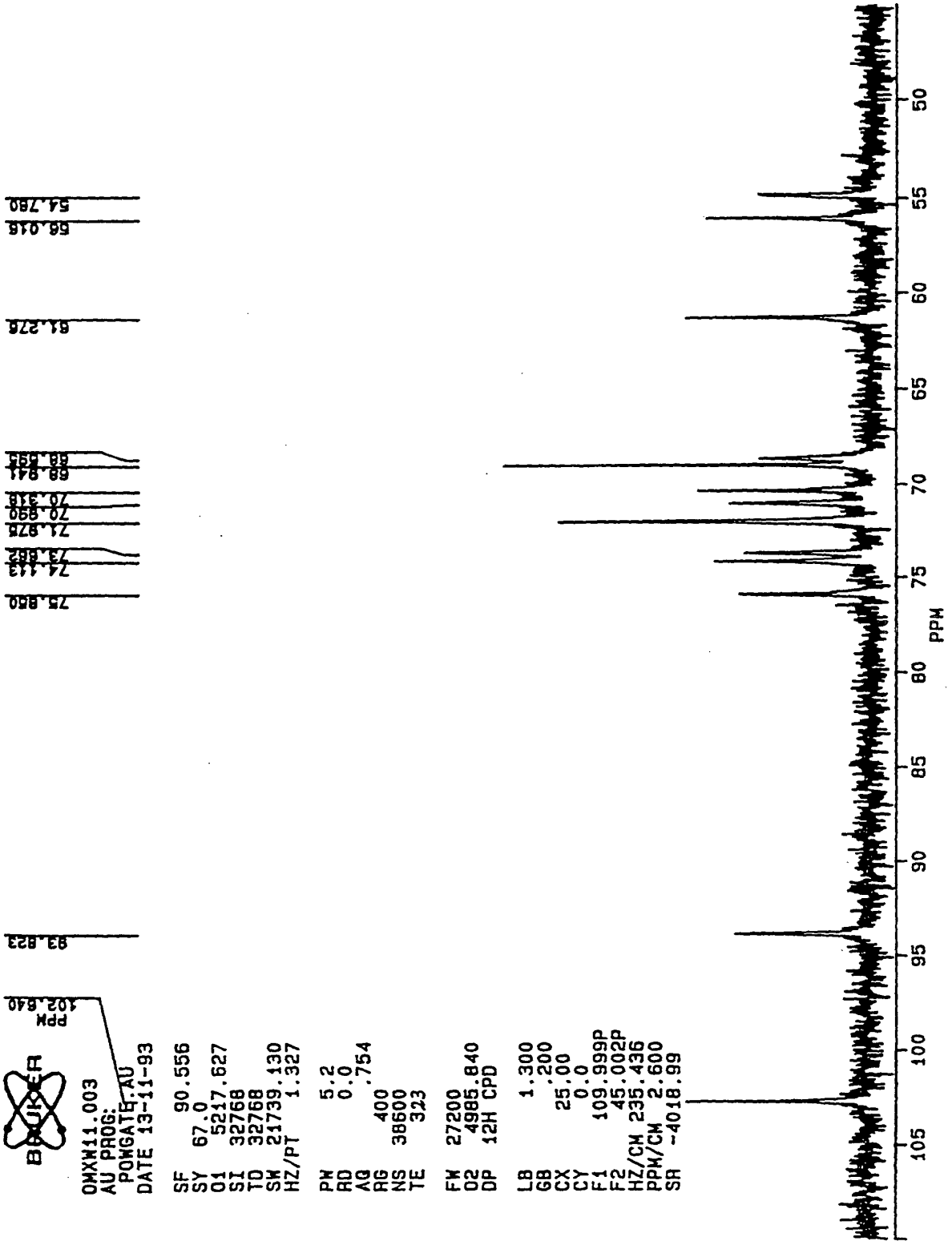

Fig. 5

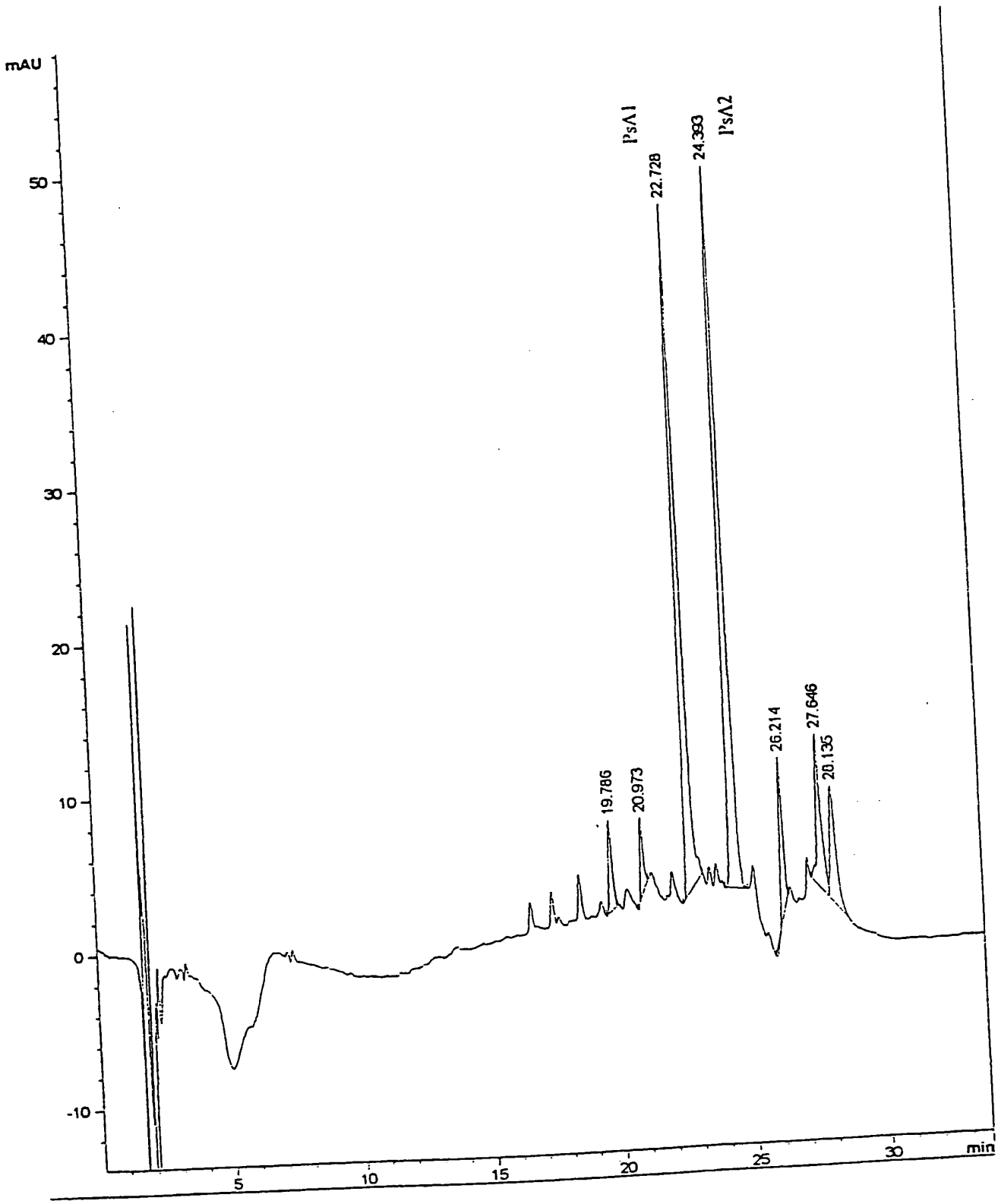

Fig. 6

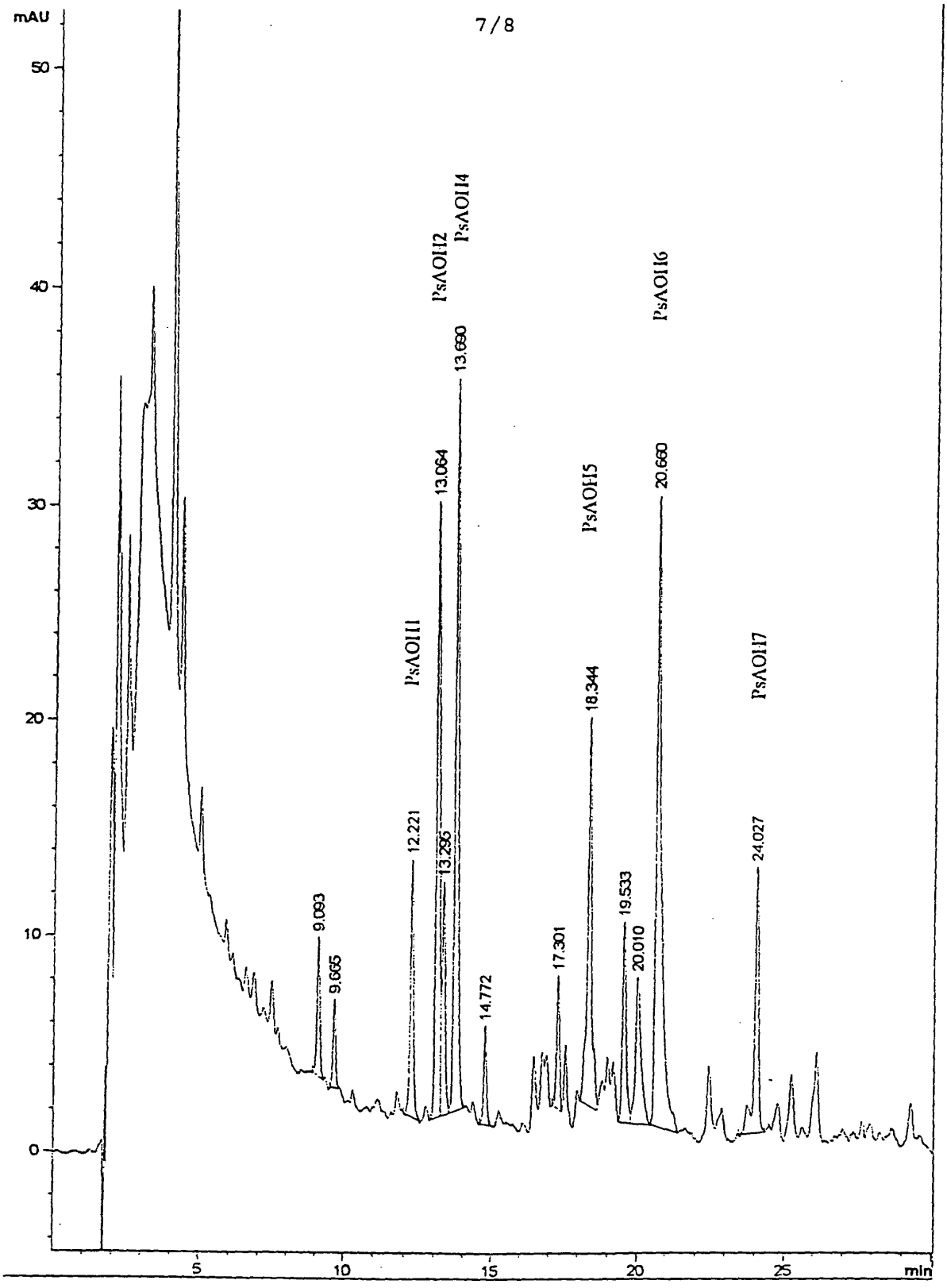

Fig. 7

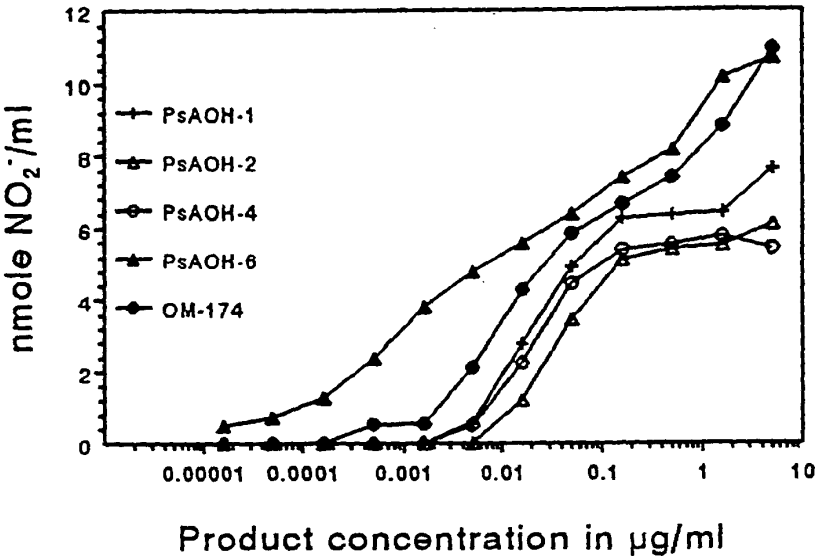

Fig. 8

INTERNATIONAL SEARCH REPORT

Interna[illegible]l Application No
PC[illegible] 94/03852

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H13/06 C07H15/04 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS I, no.10, 1984, LETCHWORTH GB pages 2291 - 2295 D. CHARON ET AL 'Chemistry of bacterial endotoxins. Part 2. A practical synthesis of 6-O-{4-O-ammonio(hydrogen)phosphono-2-deoxy-2-[(3R)-3-hydroxytetradecanamido]-beta-D-glucopyranosyl}-2-deoxy-2-[(3R)-3-hydroxytetradecanamido]-D-glucose.' see page 2291 - page 2293 ---	1,2,4-6, 9-11, 13-16, 19-23, 27-33
A	EP,A,0 192 296 (AKZO N. V.) 27 August 1986 see claims; examples --- -/--	1,27, 31-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 1995	21. 03. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TETRAHEDRON LETTERS, vol.26, no.7, 1985, OXFORD, GB pages 909 - 912 S. KUSUMOTO ET AL 'Chemical synthesis of 1-dephospho derivative of Escherichia coli lipid A' see the whole document	1,27, 31-33
A	BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, vol.60, no.6, 1987, TOKYO, JP pages 2205 - 2214 M. IMOTO ET AL 'Total synthesis of Escherichia coli lipid A, the endotoxically active principle of cell-surface lipopolysaccharide' see the whole document	1,27, 31-33

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0192296	27-08-86	AU-B- 583093	20-04-89
		AU-A- 5348886	28-08-86
		CA-A- 1259308	12-09-89
		JP-A- 61246195	01-11-86
		US-A- 4719202	12-01-88

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(71) Applicant: **SUNTORY KABUSHIKI KAISHA**
1-40, Dojimahama 2-chome
Kita-ku
Osaka (JP)

(72) Inventor: **KODAMA, Tohru**
26-21, Higashikanmaki 2-chome
Takatsuki-shi
Osaka 569 (JP)
Inventor: **SAITOH, Masayuki**
18-7-301, Inaba-cho
Ibaraki-shi
Osaka 567 (JP)
Inventor: **OGAWA, Tomohiko**
1-8, Okamachi-kita 1-chome
Toyonaka-shi
Osaka 560 (JP)

(74) Representative: **Hansen, Bernd, Dr.**
Dipl.-Chem. et al
Hoffmann, Eitle & Partner,
Patentanwälte,
Arabellastrasse 4
D-81925 München (DE)

(54) **NOVEL DISACCHARIDE DERIVATIVE.**

(57) A novel disaccharide derivative represented by chemical formula (I), stereochemical isomers and salts thereof, and a pharmaceutical composition containing the same as the active ingredient. The compound has various biological activities such as potent mitogenic activity, adjuvant activity, polyclonal B-cell activating (nonspecific protective) activity, natural killer activity, antitumor activity and antiviral activity, but scarcely has the activity of inducing the production of so-called inflammatory cytokines, such as tumor necrotizing factor (TNF) and IL-1, from macrophages unlike the lipid A and derivatives thereof. Therefore, it is free from noxious effects in which the lipid A and derivatives thereof have been problematic, such as lethal toxicity and pyrogenicity, and hence it is useful not only as immunopotentiator, antitumor agent and antiviral agent, but also as a therapeutic or preventive agent for sepsis, rheumatoid arthritis, and so forth.

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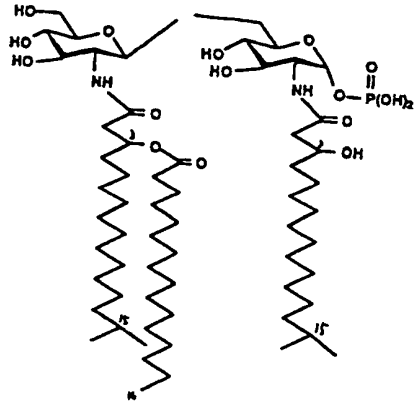


[Technical Field]

This invention relates to a novel disaccharide derivative, which has various biological activities and a low toxicity (i.e., extremely low lethal toxicity and pyrogenicity), and its salt.

[Background Art]

Lipopolysaccharide (LPS), which is contained in the outer membrane of the cell wall of various gram-negative bacteria, consists of a glycolipid called "lipid A" to which various saccharides are bonded. It has been known for a long time that LPS is the main component of endotoxins. It is also known that LPS accelerates various immune functions *in vivo* and its main activity expression site resides in lipid A. It is understood that LPS has various biological activities in addition to an immunomodulatory effect and an antitumor effect.

The chemical structure of lipid A has been clarified in various gram-negative bacteria including *Escherichia coli* ["Structure of the lipopolysaccharide from an E. coli Heptose-less Mutant", Marcha R. R., Jiunn-yann T., Israel B., and H. Gobind Khorana, The Journal of Biological Chemistry, vol. 254, No. 13, pp. 5906 - 5917 (1979)]. Among all, the chemical synthesis of lipid A originating in *Escherichia coli* has been completed and various derivatives thereof are also chemically synthesized. As a result, it is proven that some of chemically synthesized lipid A derivatives are comparable or even superior to the lipid A originating in *Escherichia coli* in the function of inducing tumor necrosis factor (TNF) and mitogen activity [Japanese Patent Application Laid-Open (Kokai) No. Sho-59-48497].

However, the lipid A originating in *Escherichia coli* and its derivatives exhibit some unfavorable properties such as pyrogenicity and necrotoxic activity. Thus attempts were made to synthesize lipid A derivatives over an extended range [Japanese Patent Application Laid-Open (Kokai) No. Sho-61-227586]. Further, detailed studies were conducted on compounds having a monosaccharide structure with lipid A-like activities, modification with the use of various substituents and substituent-introduction sites. Also various analogs were synthesized and the biological activities, immunological activities and toxicities of these substances were examined ["Ripido A Ruijitai no Seibutsu Kassei (Biological Activities of Lipid A Analogs)", Ogawa H., Kiso M. and Hasegawa A., Taisha (Metabolism), vol. 26, No. 5, pp. 15 - 27 (1989); and "Gosei Ripido A to sono Yudotai (Synthetic Lipid A and its Derivatives)", Honma Y., Meneki Yakuri (Immunopharmacology), vol. 8, No. 4, pp. 25 - 32 (1990)]. However, no reference has been made concerning a compound having free hydroxyl groups at the 3, 3' and 4'-positions and no compound practically available as a medicine has been developed so far.

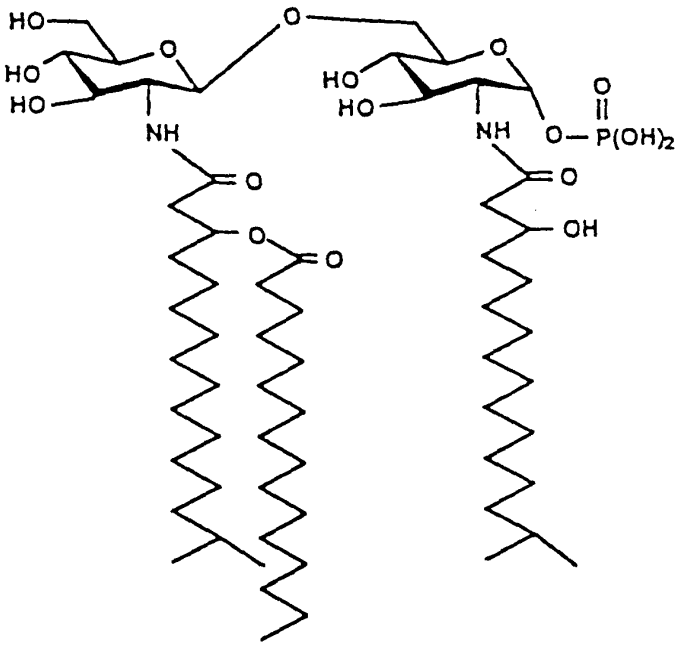
[Disclosure of the Invention]

Under these circumstances, it has been strongly desired to develop a lipid A analog which has a reduced toxicity and enhanced activities.

The present invention provides a novel disaccharide derivative which has various useful biological activities, for example, potent mitogen activity, adjuvant activity, nonspecific protective activity, antiviral activity, immunopotentiation function, etc. but little adverse effects, for example, pyrogenicity, lethal toxicity, etc. and is highly useful as a medicine, etc.

The present inventors have found that LPS contained in the outer membrane of cell wall of *Porphyromonas (Bacteroides) gingivalis*, which is one of bacteria commonly found in human oral cavity and seemingly being causative of periodontal diseases, has mitogen activity, etc. but yet extremely low lethal toxicity and pyrogenicity. They have further prepared and purified the activity expression site of this LPS, analyzed its structure and effected extensive studies thereon. As a result, they have found that the active compound of the present invention has a glucosamine β(1,6)-disaccharide structure having a phosphate group bonded to the 1-position as the basic skeleton and 3-hydroxy-15-methylhexadecanoic acid is bonded to the amino group at the 2-position thereof via an amide linkage while 3-hexadecanoyloxy-15-methylhexadecanoic acid is bonded to the amino group at the 2'-position thereof via an amide linkage. Thus the structure of the compound of the present invention is characterized in that it has no phosphate group at the 4'-position and the hydroxyl groups at the 3- and 3'-positions remain in a free state, largely differing from the conventional lipid A derivatives. It is therefore assumed that the compound of the present invention has a structur represented by the following Formula I.

[Formula I]



It has been further found that the compound of the present invention has various biological activities, for example, potent mitogen activity, adjuvant activity, polyclonal B cell activation (nonspecific protective) activity and natural killer activity, but yet little activity of inducing the production of so-called inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1 from macrophages as observed in the conventional lipid A and its derivatives. Accordingly, the compound of the present invention is useful as an immunopotentiator being free from any adverse effects such as lethal toxicity or pyrogenicity which arise in the case of the conventional lipid A and its derivatives.

It has been further found that the compound of the present invention suppresses the production of IL-1, which is induced by lipid A of *Escherichia coli*, and induces the production of IL-1 receptor antagonist (IL-1ra). Together with the potent nonspecific protective activity as described above, these activities make the compound useful as an agent for preventing and treating pathologic conditions induced by the infection with gram-negative bacteria such as *Escherichia coli*, in particular, sepsis or septic shock. Because of being capable of suppressing the production of IL-1 and inducing the production of the IL-1 receptor antagonist (IL-1ra), furthermore, the compound of the present invention is useful as a remedy for pathologic conditions accompanied by the abnormal production of IL-1 per se, for example, chronic rheumatoid arthritis, etc.

It has been also found that this compound activates natural killer cells and shows an antitumor activity and an antiviral activity. The antitumor activity suggests that it is useful as an antitumor agent, while the antiviral activity and the potent nonspecific protective activity indicate its usefulness as an antiviral agent.

Based on these characteristics, it is expected that the novel disaccharide derivative according to the present invention or its salt is particularly useful in a medicinal composition which comprises this compound together with pharmaceutical carriers and/or diluents.

Although the compound of the present invention may exist in the form of various stereoisomers, individually isolated isomers and isomeric mixtures are all involved in the technical idea of the present invention. The compound of the present invention can be prepared and purified from a microbial source and then used. Alternatively, it can be produced by various chemical synthesis techniques.

Now specific examples of the production of the compound of the present invention by each method will be given.

(1) Production example with the use of microorganism

In order to produce the compound of the present invention from a microorganism, any microorganism can be used so long as it can produce the compound of the present invention represented by the above Formula I. For example, *Porphyromonas (Bacteroides) gingivalis* (ATCC Catalogue No. 33277) is usable therefor.

Although either a liquid medium or a solid one may be used in the incubation, it is usually convenient to effect anaerobic stationary culture in a liquid medium. Any medium may be employed, so long as the microorganism producing the compound of the present invention can grow and produce the compound of the present invention therein. The conditions for incubation (temperature, period, properties of medium, etc.) may be appropriately selected and controlled in such a manner as to give the maximum yield of the compound of the present invention. It is preferable that the incubation is effected under anaerobic conditions at a temperature of 25 to 40 °C, still preferably at 37 °C, for 12 to 36 hours, still preferably for 26 hours and the pH value of the medium is maintained at 6.0 to 8.0, still preferably at 7.3.

The compound of the present invention is produced and accumulated by incubating the microorganism under the above-described conditions. Then the cells are collected from the culture medium by filtration or centrifugation and the target compound is separated and purified therefrom. To separate and purify LPS from the cells, various means may be selected depending on the chemical properties of the compound. For example, LPS can be separated and purified by appropriately combining separation and purification techniques, for example, extraction with hot phenol-water, treatment with various enzymes, centrifugation, solvent fractionation and column chromatography with the use of various resins. After repeatedly purifying, the LPS or crude LPS thus obtained is hydrolyzed with a weak acid. This hydrolysis may be carried out by any method, so long as the compound of the present invention is thus liberated. Preferably, the hydrolysis is effected with the use of 0.05 to 0.2N acetic acid at a temperature of 90 to 110 °C for 2 to 3 hours. The target compound may be separated and purified from the reaction mixture by various techniques selected depending on the chemical properties of the compound of the present invention. Namely, solvent fractionation and column chromatography with the use of various resins may be employed. By appropriately combining these techniques, the compound of the present invention can be separated and purified.

(2) Production example via chemical synthesis

An N-glucosamine derivative, which has been protected with an appropriate protecting group at an appropriate position, is converted into a disaccharide derivative via a glycosidation reaction. Then the disaccharide derivative is N-acylated with a fatty acid and phosphorylated at the 1-position at the reducing end followed by deblocking. Alternatively, a protected N-glucosamine derivative, which has been N-acylated with a desired fatty acid, is converted into a disaccharide derivative via a glycosidation reaction. Then the disaccharide derivative is phosphorylated and deblocked.

The compound of the present invention thus obtained is a compound capable of forming a salt at its phosphate moiety. Therefore it can be easily converted into a salt by a publicly known method. Examples of such salts include alkali metal salts for example, sodium salt, potassium salt, etc.; alkaline earth metal salts for example, calcium salt, magnesium salt, etc.; ammonium salts and pharmaceutically acceptable amine salts. Examples of nontoxic amine salts include tetraalkylammonium salts for example, tetramethylammonium salt, etc.; and organic amine salts for example, methylamine salt, triethylamine salt, cyclopentylamine salt, benzylamine salt, pyridine salt, piperidine salt, diethanolamine salt, lysine salt, arginine salt, etc.

The disaccharide derivative, i.e. the compound of the present invention, or its salt thus obtained may be administered in the form of a medicinal composition for therapeutic or preventive purposes either systemically or topically and either orally or parenterally. Although the administration dose varies depending on age, body weight, conditions, administration route, etc., it is usually administered to an adult in a single dose of from 0.01 to 100 mg once to several times per day either orally or parenterally. As a matter of course, the dose varies depending on various factors. Thus a satisfactory effect can be achieved in some cases by administering the compound in a smaller dose than the lower limit as specified above, while it is sometimes needed to administer the compound in a dose xceeding the upper limit as specified above.

A solid medicinal composition of the present invention for oral administration includes tablets, powders granules, etc. In such a solid composition, the compound of the present invention is mixed with at least one inert diluent, for example, lactose, glucose, microcrystalline cellulose, starch, polyvinyl pyrrolidone, magnesium metasilicate aluminate, etc. In addition to the inert diluent, the composition may contain other additives such as a lubricant for example, magnesium stearate or a disintegrating agent for example, cellulose

calcium gluconate. Tablets or pills may be coated with a gastric or enteric coating film made of, for example, sucrose, gelatin, hydroxypropylcellulose, etc., if necessary. Also, they may be coated with two or more layers. It is also possible to use capsules made of a material such as gelatin which can be taken up by human body.

A liquid medicinal composition for oral administration includes pharmaceutically acceptable emulsions, solutions, suspensions and syrups. Examples of inert diluents generally employed in the art therefor include purified water, ethanol, etc. In addition to the inert diluent, the liquid composition may contain auxiliaries such as a humectant, a suspending agent, etc. and additives such as a sweetening agent, a flavor, an antiseptic, etc. The composition for oral administration also includes sprays which are formulated in a conventional manner. An injection composition of the present invention for parenteral administration includes sterile aqueous or nonaqueous solutions, suspensions and emulsions. Aqueous solutions and suspensions contain, for example, distilled water for injection and physiological saline. Nonaqueous solutions and suspensions contain, for example, propylene glycol, polyethylene glycol, vegetable oils such as olive oil, alcohols such as ethanol, Polysorbate 80 (registered trademark), etc. Such a composition may further contain auxiliaries such as an antiseptic, a humectant, an emulsifier or a dispersion aid. These compositions are sterilized by a specific filtration technique, addition of a bactericide or irradiation. It is also possible to prepare a sterile solid composition which is dissolved in sterile water or a sterile solvent for injection before use. Compositions for parenteral administration also involve liquid preparations for external use, embrocations such as ointments, suppositories, pessaries, etc. each formulated by a publicly known method.

It has been found that the compound of the present invention has various biological activities, for example, potent mitogenic activity, adjuvant activity, polyclonal B cell activation (nonspecific protective) activity, natural killer activity, etc., but little activity of inducing the production of so-called inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1 from macrophages as observed in the conventional lipid A and its derivatives. Accordingly, the compound of the present invention is free from any adverse effects such as lethal toxicity or pyrogenicity, which cause troubles in the conventional lipid A and its derivatives, and thus useful as an immunopotentiator.

It has been further found that the compound of the present invention suppresses the production of IL-1 induced by lipid A of *Escherichia coli* and induces the production of IL-1 receptor antagonist (IL-1ra). Together with the potent nonspecific protective activity, these activities make the compound of the present invention useful as an agent for preventing and treating pathologic conditions induced by the infection with gram-negative bacteria such as *Escherichia coli*, in particular, sepsis or septic shock. Due to its ability to suppress the production of IL-1 and inducing the production of the IL-1 receptor antagonist (IL-1ra), furthermore, the compound of the present invention is useful as a remedy for pathologic conditions accompanied by the abnormal production of IL-1 per se, for example, chronic rheumatoid arthritis, etc.

It has been also found that this compound activates natural killer cells and shows an antitumor activity and an antiviral activity. The antitumor activity suggests that it is useful as an antitumor agent, while the antiviral activity and the potent nonspecific protective activity indicate its usefulness as an antiviral agent.

Based on these characteristics, it is expected that the novel disaccharide derivative according to the present invention or its salt is particularly useful in a medicinal composition which comprises this compound together with pharmaceutical carriers and/or diluents.

Now the compound according to the present invention will be described in greater detail. However, it is to be understood that the present invention is not restricted to the specific embodiments described therein.

Example 1: Production from microorganism

Porphyromonas (Bacteroides) gingivalis was anaerobically incubated in 160 l of a GAM bouillon (manufactured by Nissui Seiyaku K.K.) medium (pH 7.3) at 37 °C for 26 hours. After the completion of the incubation, the cells were collected by centrifuging the culture medium and freeze-dried. Thus 100 g of dry cells were obtained. These dry cells were extracted by the hot phenol-water extraction method to thereby give crude LPS. Namely, 3.5 l of distilled water was added to 100 g of the dry cells and heated to 68 °C. Separately, 90% phenol was heated to 68 °C and added thereto. The resulting mixture was then stirred at 68 °C for 20 minutes, cooled with ice and centrifuged. The aqueous layer was collected and 3.5 l of distilled water was added again. After repeating the extraction procedure, the aqueous layers thus obtained were combined, sufficiently dialyzed against distilled water, concentrated and freeze-dried. Thus 12.47 g of a crude extract was obtained. Ten g of this crude extract was suspended in 1 l of distilled water and ultracentrifuged. The precipitate was treated with Nuclease P1 (manufactured by Yamasa Shoyu K.K.) and Pronase (manufactured by Calbio-chemical, U.S.A.) twice for each enzyme. Then the above-described ultracentrifugal was washed twice with distilled water and the precipitate was freeze-dried to thereby give

250 mg of a crude LPS fraction. The crude LPS fraction (250 mg) was suspended in a 50 mM Tris-HCl buffer (pH 7.4) and subjected to Sepharose 4B column chromatography (inner diameter: 1.5 cm, height: 90 cm). The excluded volume fraction was collected, precipitated from ethanol, washed with distilled water twice and then freeze-dried. Thus 110 mg of an LPS fraction was obtained. This LPS fraction was hydrolyzed with a weak acid (0.1N acetic acid) at 105°C for 2.5 hours. Then the reaction mixture was centrifuged to give a precipitate. This fraction was purified by silica gel column chromatography (chloroform/methanol/water/triethylamine = 30/12/1.5/0.1). Thus 4.5 mg of the compound of the present invention was obtained.

Example 2: Analysis of structure

The physicochemical properties of the compound obtained in the above Example 1 were examined. The results are as follows:

(1) analysis on saccharides and fatty acids:

a. having, as the basic skeleton, a glucosamine β-(1-6)disaccharidestructure, to which a phosphate group is attached to the 1-position via an ester linkage;

b. having 3-hydroxy-15-methylhexadecanoic acid attached to the amino group at the 2-position via an amide linkage;

c. having 3-hexadecanoyl-15-methylhexadecanoic acid attached to the amino group at the 2'-position via an amide linkage;

d. having no phosphate group at the 4'-position; and

e. the hydroxyl groups at the 3-, 3'- and 4'-positions remaining in a free state;

(2) molecular formula:

$C_{62}H_{119}O_{17}N_2P$;

(3) color change reaction:
being positive in the sulfuric reaction, positive in the Dittmer-Lester reaction, negative in the ninhydrin reaction and negative in the TTC reaction;

(4) color and form:
a white powder;

(5) mass spectrum:
negative FAB-MS-MS, M/Z 1193 (M-H), 937 (M-2H-$C_{15}H_{31}COO$);

(6) NMR spectrum:
^{1}H-NMR (300 MHz, $CDCl_3$ + MeOD + D_2O) δ: 0.81 (12H,d), 0.82 (3H,t), 1.1-1.6 (72H,m,CH_2,CH), 2.2-2.5 (6H,m,CO-CH_2), 3.1-4.3 (13H,m), 4.46 (1H,d), 5.15 (1H,m), 5.45 (1H,m).

Based on these results, it was assumed that the structure of compound of the present invention is the one represented by Formula I.

Example 3: Measurement of activity

The results of the measurement of the physiological activities of the compound of the present invention will be shown below.

(1) Mitogenic activity

The mitogenic activity was measured by, for example, determining the amount of ^{3}H-thymidine incorporated into isolated and cultured mouse lymphoid cells. Namely, the spleen of a BALB/c mouse was ground and 5×10^5 cells/well (200 μl) of these spleen cells were incubated in the presence of the compound of the present invention at definite concentrations, in the presence of a comparative compound, or in the medium alone. Six hours before the completion of the incubation, 37 kBq/well (10 μl) of ^{3}H-thymidine was added. After the completion of the incubation, the amount of ^{3}H-thymidine (radioactivity) incorporated into the cells was determined. The results are expressed in "Stimulation Index" calculated in accordance with the following formula.

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$$\text{Stimulation Index} = \frac{\text{Radioactivioty (cpm) of test group}}{\text{Radioactivity (cpm) of control group (medium alone)}}$$

As Table 1 shows, the compound of the present invention has a mitogen activity. As the comparative compound, synthetic lipid A 506 was employed. This compound 506 is 6-O-[2-deoxy-2-(3-dodecanoyloxytetradecanoylamino)-3-O-(3-tetra-decanoyloxytetradecanoyl)-4-O-phosphono-β-D-glucopyranosyl]-2-deoxy-2-(3-hydroxytetradecanoylamino)-3-O-tetradecanoyl-1-O-phosphono-α-D-glucopyranose.

Table 1

Mitogen activity		
Compound		Stimulation Index
Invention compound	none	1.00
Invention compound	5 μg/ml	4.17
Invention compound	50 μg/ml	7.46
Comparative compound 506	50 μg/ml	6.28

(2) NK (natural killer) activity

This test was carried out in the following manner. Namely, 100 μg of the compound of the present invention or the comparative compound was intravenously injected into a BALB/c mouse on the days 0 and 7. On the day 14, the spleen cells of the animal were collected. To the prepared spleen cells (1×10^6 cells/0.1 ml), target cells (Moloney virus-induced lymphoma YAC-1; 2×10^4 cells/ml) labeled with ^{51}Cr were added followed by the incubation for 4 hours. The medium containing no spleen cell was added to the minimum free control group, while 0.1N NaOH was added to the maximum free control group. After the completion of the incubation, 0.1 ml of the culture supernatant was collected and the amount (radiation dose) of ^{51}Cr liberated due to the damage in the target cells was measured. Then the NK activity (%) was calculated by substituting each value in accordance with the following formula.

NK Activity (%) = {(Radioactivity of test group - Radioactivity of minimum free control group)(cpm) / (Radioactivity maximum free control group - Radioactivity of minimum free control group)(cpm)}

As the result of the calculation with the use of the above formula, the compound of the present invention shows an NK activity of 50.1%. Thus it is proved to be comparable to the synthetic lipid A 506 in this activity.

(3) Antitumor activity

This test was effected by examining a cytostatic activity on methylcholanthrene-induced fibrosarcoma (Meth A). Namely, adherent cells (macrophages) were collected from spleen cells of a BALB/c mouse. To the macrophages thus prepared (2×10^5 cells/ml) and target cells (Meth A: 2×10^4 cells/0.1 ml), the compound of the present invention or the comparative compound was added to give a definite concentration. After incubating for 18 hours, 14.8 kBq/well (10 μl) of ^3H-thymidine was added and the incubation was continued for additional 6 hours. After the completion of the incubation, the amount (radioactivity) of ^3H-thymidine thus incorporated into the cells was measured. Then the ^3H-thymidine-uptake suppression ratio was calculated by substituting the obtained values in accordance with the following formula and the result was expressed as the cytostatic activity on macrophages.

Cytostatic Activity (%) = {1-(Radioactivity of macrophages and target cells - Radioactivity of macrophages alone)(cpm) / (Radioactivity of target cells along)} × 100

The compound of the present invention showed the data as given in Table 2. Namely, it exhibited a cytostatic activity comparable to that of the synthetic lipid A 506.

Table 2

Compound		Cytostatic activity
Invention compound	100 μg/ml	64.0
Invention compound	10 μg/ml	58.6

(4) Adjuvant activity

In this test, male BALB/c mice (each group having 6 animals) were used. On the days 0 and 28, 100 μg of bovine serum albumin (BSA) containing 100 μg of the compound of the present invention on the comparative compound or no such a compound was subcutaneously injected into each animal in the form of a water-in-oil type emulsion in Freund's incomplete adjuvant (FIA). On the day 5 following the booster, the level of anti-BSA IgG antibody formed in the serum was determined by the ELISA method. The result was expressed in "Stimulation Index" calculated in accordance with the following formula.

$$\text{Stimulation Index} = \frac{\text{Antibody level achieved by adding compound and BSA to FIA } (\mu g/ml)}{\text{Antibody level achieved by adding BSA alone to FIA } (\mu g/ml)}.$$

As Table 3 shows, it was found that the compound of the present invention is superior in the adjuvant activity to the synthetic lipid A 506.

Table 3

Compound	Stimulation Index
BSA alone	1.00
Invention compound	2.48
Comparative compound 506	1.99

(5) Antiviral activity

In this test, suppression of the effect of vesicular stomatitis virus (VSV) on a mouse fibroblast line L929 was employed as an indication. Namely, 4×10^4 cells/0.1 ml of L929 cells were added to each well and incubated for 24 hours. Then 0.1 ml portions of diluted samples of the compound of the present invention or the comparative compound (1 mg/ml) from the serial dilution systems were added thereto and the incubation was continued for additional 24 hours. After discarding the culture supernatant, VSV adjusted to 100 $TCID_{50}$/0.1 ml was added and incubated for 24 hours. Then the culture medium was eliminated and fixed with a 5% solution of formaldehyde for 20 minutes. It was then stained with a 0.5% solution of Crystal Violet for 20 minutes. After washing with water and drying, the absorbance was measured at 600 nm. The activity was expressed in the reciprocal of the dilution ratio to the sample concentration (1 mg/ml) of the original sample solution wherein L929 cells survived at a ratio of 50%.

As Table 4 shows, it was found that the compound of the present invention has an antiviral activity stronger than that of the synthetic lipid A 506.

Table 4

Antiviral activity	
Compound	Antiviral activity
Invention compound	4.9
Comparative compound 506	2.2

(6) Polyclonal B cell activation activity

In this test, the activity was examined by using the ELISPOT (Enzyme-Linked Immunospot) method. BALB/C mouse spleen cells (2.5×10^6 cells) were incubated in RPMI 1640 medium containing 5% of fetal bovine serum (FBS) at 37 °C for 72 hours in the presence of a definite amount of the compound of the present invention or the comparative compound or in the absence of such a compound. After washing, the antibody-producing cells were counted by the ELISPOT method.

Namely, the above-described spleen cells were added to each well of a plate, which had been coated with goat antimouse immunoglobulin and treated with 5% FBS, and incubated for 4 hours. After washing away the cells, the plate was reacted with goat antimouse μ-chain specific antiserum labeled with biotin at 25 °C overnight, washed with physiological buffer saline (PBS) and then treated with peroxidase-labeled streptoavidin. The activity was determined by counting the spots (cells) formed by the antibody-producing cells under a stereoscopic microscope and expressed in the stimulation index, i.e., the ratio of the cell count in the presence of the test compound to the cell count in the absence of the same (control). The concentration of the test compound was expressed in μg per 2.5×10^6 cells.

As Table 5 shows, it was found out that the compound of the present invention is comparable or even superior to the synthetic lipid A 506 in the activity of activating polyclonal B cells. Thus it seemingly has a potent nonspecific protective activity.

Table 5

Polyclonal B cell activation activity		
Compound		Stimulation Index
Invention compound	none	1.0
Invention compound	100 μg	30.3
Invention compound	10 μg	22.0
Comparative compound	100 μg	20.7
Comparative compound	10 μg	15.7

(7) Cytokine inducing activity

The cytokine inducing activity was tested by using an ELISA system for assaying TNF-α (manufactured by Amersham Japan) and another ELISA system for assaying IL-1β (manufactured by Otsuka Pharmaceutical Co., Ltd.). Namely, human peripheral blood monocytes (5×10^5) were incubated in the presence of a definite concentration of the compound of the present invention or the comparative compound for 24 hours. Then the cytokines in the culture supernatant were assayed by the ELISA method.

As a result, the compound of the present invention showed little activity of inducing the production of TNF-α or IL-1β from human peripheral blood monocytes. It was also found that when the compound of the pr sent invention (50 times as much) was added simultaneously with the synthetic lipid A 506 or LPS originating in *Escherichia coli*, the invention compound suppressed the production of IL-1β induced by the compound 506 or the LPS originating in *Escherichia coli*.

By using an ELISA system (manufactured by R & D) for assaying IL-1 receptor antagonist (IL-1ra), it was further found that the compound of the present invention produced IL-1ra in the culture supernatant of

human peripheral blood monocytes in a larger amount than the synthetic lipid A 506 did.

(8) Galactosamine-loaded lethal toxicity test

The galactosamine-loaded lethal toxicity was determined by using the following experimental system.

16 mg of D-galactosamine/HCl was intraperitoneally administered to a male C57BL mouse aged 8 weeks. Immediately thereafter, the compound of the present invention was intravenously injected into the animal and the conditions were observed after 24 hours.

The compound of the present invention showed the activity as shown in Table 6 and, therefore, was proved to be less toxic.

Table 6

Galactosamine-loaded lethal toxicity	
Compound	LD_{50}
Invention compound	> 10 μg
Comparative compound 506	0.0079 μg

(9) Other toxicities

(i) Local Shwartzman reaction

This test was carried out in the following manner. The test compound, which had been diluted to a definite concentration with 0.2 ml of physiological saline, was intradermally injected into a male rabbit. After 24 hours, 100 μg/ml/kg of *Salmonella minnesota* 9700 LPS-W (manufactured by Difco) was intravenously injected into the animal for elicitation. After 4 hours, intradermal hemorrhage was observed. As a result, the compound of the present invention caused no hemorrhage in a dose of 100 μg/site.

(ii) Pyrogenicity test

In this test, rabbits were used and 5 ml/kg of the test compound diluted to a definite concentration with physiological saline was intravenously injected into each animal. Then the rectal temperature was measured. Rabbits showing an increase in the bodily temperature by 0.6 °C or more were referred to as feverish. As a result, the synthetic lipid A 506 showed pyrogenicity in a dose of 0.01 μg, while the compound of the present invention showed no pyrogenicity even in a dose of 10 μg/kg.

(iii) *Limulus* test

In this test, Pregel (manufactured by Seikagaku Kogyo K.K.), i.e., a reagent for assaying an endotoxin was used. By using a freeze-dried product prepared from a *Tachypleus tridentatus* lysate, the ability to form a gel was examined. As a result, the minimum effective dose of the compound of the present invention was 1,000 times as much as that of the synthetic lipid A 506, which indicates that the invention compound has a markedly low toxicity.

That is to say, the compound of the present invention shows little toxicity in various tests including the Limulus test, the local Shwartzman reaction and the pyrogenicity test.

Claims

1. A novel disaccharide derivative showing the following physical data:
   (1) color reaction:
   being positive in the sulfuric reaction, positive in the Dittmer-Lester reaction, negative in the ninhydrin reaction and negative in the TTC reaction;
   (2) NMR spectrum:
   ^{1}H-NMR (300 MHz, $CDCl_3$ + MeOD + D_2O) δ: 0.81 (12H,d), 0.82 (3H,t), 1.1-1.6 (72H,m,CH_2,CH), 2.2-2.5 (6H,m,CO-CH_2), 3.1-4.3 (13H,m), 4.46 (1H,d), 5.15 (1H,m), 5.45 (1H,m); and

(3) mass spectrum:
negative FAB-MS-MS, M/Z 1193 (M-H), 937 (M-2H-$C_{15}H_{31}COO$);
or its salt.

2. A novel disaccharide derivative;
(1) having, as the basic skeleton, a glucosamine β-(1-6)disaccharide structure, to which a phosphate group is attached to the 1-position via an ester linkage;
(2) having 3-hydroxy-15-methylhexadecanoic acid attached to the amino group at the 2-position via an amide linkage;
(3) having 3-hexadecanoyl-15-methylhexadecanoic acid attached to the amino group at the 2'-position via an amide linkage;
(4) having no phosphate group at the 4'-position; and
(5) the hydroxyl groups at the 3-, 3'- and 4'-positions remaining in a free state;
or its salt.

3. A medicinal composition comprising a compound as claimed in Claim 1 or 2 or a pharmaceutically acceptable salt thereof together with pharmaceutical carriers and/or diluents.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP94/00376

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^5 C07H11/04, A61K37/20, C12P19/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^5 C07H11/04, A61K37/20, C12P19/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 59-48497 (Daiichi Seiyaku Co., Ltd.), March 19, 1984 (19. 03. 84), (Family: none)	1-3
A	JP, A, 61-227586 (Daiichi Seiyaku Co., Ltd.), October 9, 1986 (09. 10. 86), (Family: none)	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
May 27, 1994 (27. 05. 94)	June 14, 1994 (14. 06. 94)
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(71) Applicant: **Toho Yakuhin Kogyo Kabushiki Kaisha**
Yachiyo Building 1-14, Awaji-machi
Higashi-ku Osaka-shi Osaka-fu(JP)

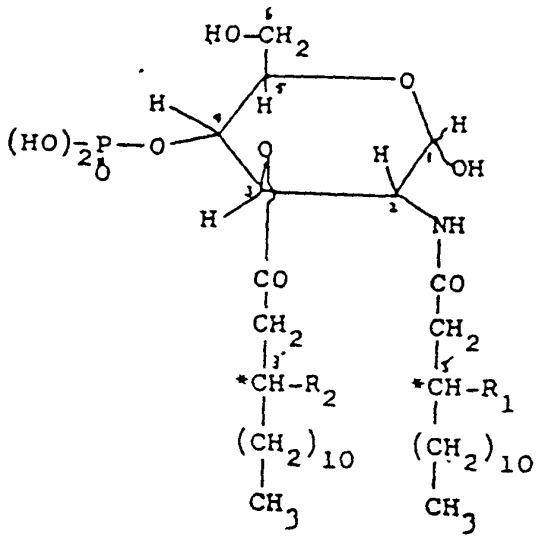
(72) Inventor: **Hasegawa, Akira**
1735-160, Kano'okurayama
Gifu-shi Gifu-ken(JP)
Inventor: **Kiso, Makoto**
57-47, Monju, Motosu-cho
Motosu-gun Gifu-ken(JP)
Inventor: **Morihara, Kazuyuki**
5-9-2, Hirose Shimamoto-Cho
Mishima-gun Osaka-fu(JP)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4 P.O. Box 86 07 67
D-8000 München 86(DE)

(54) **Analogs of nonreducing monosaccharide moiety of lipid A.**

(57) Provided herein is a derivative of 2-deoxy-2-amino-4-O-phophono-D-glucopyranose, which is derived from lipid A, of the formula:

EP 0 224 260 A2



wherein R_1 and R_2 are a member in a pair selected from the group consisting of those indicated in a following table;

Compound No.	R_1	R_2
I(R,R)	$-O-CO-(CH_2)_{12}-CH_3$	$-O-CO-(CH_2)_{12}-CH_3$
II	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
II(R,R)	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
II(S,S)	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
III	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
III(R,R)	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
III(S,S)	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
IV(R)	$-H$	$-O-CO-(CH_2)_{12}-CH_3$

The compounds of this invention contain ones of a rectus and a sinister configurations and are expected to exhibit more improved biological and immunological activities than those which natural lipid A possesses originally.

" Analogs of Nonr ducing Monosaccharide Moiety of Lipid A "

BACKGROUND OF THE INVENTION

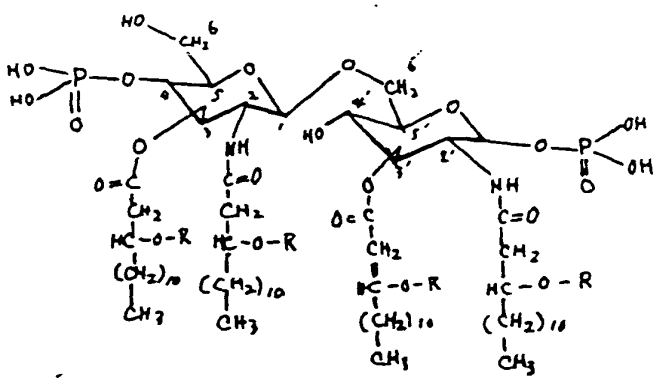
(a) Field of the Invention

This invention relates to novel analogs of the nonreducing monosaccharide subunit of lipid A and stereoisomers thereof. These novel compounds have been synthesized by the present inventors in the course of their search for an effective sugar moiety, which exhibits higher biological and immunological activities than natural lipid A.

(b) Description of the Prior Art

Lipo-polysaccharides are found in the cell-wall of some kinds of gram-negative bacilli as a main component of endotoxin. They exhibit various kinds of biological and immunological activities such as an anti-tumor activity. Lipid A is a lipoid component of lipo-polysaccharides. It is known that the biological and immunological activities of lipo-polysaccharides mostly depend on the lipid A component.

In an attempt to elucidate the chemical structure of lipid A, and to synthesize analogs of the sugar moieties of lipid A which exhibit as many biological and immunological properties of natural lipid A as possible, compounds of the following formula have been described by Galanos and Ludritz et al. in 1977 - [cf. Int. Rev. Biochem. 14: 239 (1977) and Naturwissensch. 65:578 (1987)]



wherein R represents a hydrogen atom or a straight chain aliphatic acid having 12 to 16 carbon atoms, especially myristic acid, that is tetradecanoic acid, represented by a chemical formula of $CH_3(CH_2)_{12}COOH$.

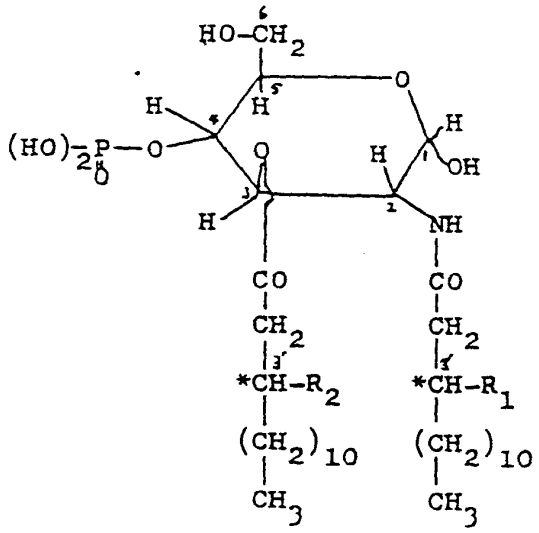
The compounds of the above structure are characterized by two glucosamine groups which are linked at their 1-and 6'-positions and the amino groups are located at the 2-and 2'-positions and the hydroxy groups are located at the 3-and 3'-positions of the glucosamine groups. Moreover, 3-hydroxy-myristic acid residues are attached by an amide or an ester linkage and the phosphoric acid groups are linked to the 1- and 4'-positions, respectively of the glucosamine groups. The compounds thus simultaneously have both hydrophilic and lipophilic substituents on the glucosamine groups.

In the above chemical formula, the left-handed glucosamine group is called the nonreducing subunit.

On the assumption that it is the nonreducing subunit which is mainly responsible for the biological and immunological activities of lipid A, the inventors have carried out an extensive research to synthesize analogs of the nonreducing sugar subunit of lipid A and the thus synthesized products have successively been subjected to a primary biological screening experim nt.

SUMMARY OF THE INVENTION

Of the large number of analogs of the nonr ducing sugar moiety of lipid A which have been synthesized by the inventors, the compounds represented be the following general formula [I] have been found to have definite biological and immunological activities, for example, inducing interferon-and tumor-necrosis factors:



[I]

wherein R_1 and R_2 are radicals whose definitions are shown in the next table:

Table

Compound No.	R_1	R_2
I(R,R)	$-O-CO-(CH_2)_{12}-CH_3$	$-O-CO-(CH_2)_{12}-CH_3$
II	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
II(R,R)	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
II(S,S)	$-O-CO-(CH_2)_{12}-CH_3$	$-OH$
III	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
III(R,R)	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
III(S,S)	$-OH$	$-O-CO-(CH_2)_{12}-CH_3$
IV(R)	$-H$	$-O-CO-(CH_2)_{12}-CH_3$

In the foregoing chemical formula and table, the carbon atoms which are marked with an asterisk* indicate an asymmetric carbon atom. Those two asymmetric carbon atoms can have a rectus configuration, hereinafter referred to as (R), or a sinister configuration, hereinafter referr d to as (S).

The preparation process of this invention is as follows: reaction of 3'-O-(substituted or non-substituted)-tetradecanoyl radical with the amino group on the C-2 position of a glucopyranose ring is carried out in the presence of dicyclohexylcarbodiimide (DCC) and reaction of the same radical with the hydroxyl group on the C-3 position is carried out in the presence of DCC or dimethylaminopyridine (DMAP). The following reactions are the protection of the hydroxyl groups on the C-4 and C-6 positions of the glucopyranose ring by coupling them with an isopropylidene group and removal thereof, protection of a hydroxyl group on the C-6 position with a trityl group and removal thereof, and reaction of a diphenylphosphono group with the hydroxyl group on the C-4 position and removal of the diphenyl group therefrom.

The inventors were the first who successfully applied these chemical reactions by properly combining them in a suitable order and manner, to the preparation of analogs of the non-reducing monosaccharide moiety of lipid A.

EXAMPLES

Example 1: Preparation of 2-Deoxy-4-O-phosphono-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[-(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucose; [Compound No. I (R,R)]

[Step a] Preparation of Benzyl 2-deoxy-4,6-O-iso-propylidene-2-[(3'R)-3'-tetradecanoyloxytetradecanamido-β-D-glucopyranoside; [Introduction of a (3R)-tetradecanoyloxytetradecanoyl group into the C-2 amino group]

Two grams of the known compound benzyl 2-amino-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside, of which preparation was published in Agric. Biol. Chem., 48, pages 251 -252 (1984) by some of the inventors et al., were dissolved in anhydrous dichloromethane (20 ml), to which (3R)-3-tetradecanoyloxytetradecanoic acid (3 g) and DCC (2.7 g) were added. The mixture was stirred for 4.5 hours at room temperature and the precipitated DCC-urea was removed by filtration. The remaining solution was washed well with dichloromethane and the filtrate and the washings were combined and concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with a mixture of dichloromethane and methanol (400 : 1) were lyophilized from 1,4-dioxane. 2.2 g (91%) of the title compound were obtained.

Melting Point: 66 -70 °C. $[\alpha]_D$ -49.3° (C = 1.127, chloroform).

Analysis (%) for $C_{44}H_{75}NO_8$ = 746.05

Calcd.: C, 70.83; H, 10.13; N, 1.88

Found : C, 70.68; H, 9.99; N, 1.82

[Step b] Preparation of Benzyl 2-deoxy-4,6-O-isopropylidene-2-[(3'R)-3'-tetradecanoyloxytetradecanamide]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-β-D-glucopyranoside; [Introduction of a (3R)-tetradecanoyloxytetradecanoyl group into the C-3 hydroxyl group]

The product of the preceding step (1.35 g) was dissolved in anhydrous dichloromethane (9 ml), to which (3R)-tetradecanoyloxytetradecanoic acid (0.82 g), DCC (0.75 g) and DMAP (0.105 g) were added. The mixture was stirred at room temperature. The completion of the reaction was confirmed by means of a thin layer chromatography (ethyl acetate : hexane = 1' : 1). The precipitated urea was removed by filtration and the remaining solution was well washed with dichloromethane. The filtrate and washings were combined and concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with a mixture of hexane and ethyl acetate (10 : 1) were lyophilized from 1,4-dioxane. 1.73 g (81 %) of the title compound were obtained.

Melting Point: 64 -65 °C. $[\alpha]_D$ -23.1° (C = 0.995, chloroform).

Analysis (%) for $C_{72}H_{127}NO_{11}$ = 1182.74

Calcd.: C, 73.11; H, 10.82; N, 1.18

Found : C, 73.38; H, 11.00; N, 1.24

[Step c] Preparation of Benzyl 2-deoxy-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-β-D-glucopyranoside [Removal of the isopropylidene group]

The product of the preceding step (1.36 g) was dissolved in 80 % acetic acid (20 ml) and the mixture was stirred for 3 hours at 45 °C. The reaction mixture was concentrated in vacuo and the residue was subjected to column chromatography (Wako gel C-200). 1.05 g (80 %) of the title compound were obtained after the elution with a mixture of dichloromethane and methanol (100:1).

DOCID <EP 0224260A2>

Melting Point: 101 -101.5 °C. $[\alpha]_D$ -16.8° (C = 0.92, chloroform).

IR$_\nu$ nujol cm^{-1} = 3600 -3200 (OH, NH), 1730 (ester), 1660, 1550 (amido), 760 -690 (ph)

[Step d] Preparation of Benzyl 2-deoxy-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradenoyl]-6-O-trityl-β-D-glucopyranoside [Tritylation of the C-6 hydroxyl group]

The product of the preceding step (0.87 g) was dissolved in pyridine (10 ml) and was stirred for 3.5 hours at 90°C. The obtained residue was dissolved in chloroform. The solution was washed with 2N hydrochloric acid and then with water and was concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with a mixture of dichloromethane and methanol (500 : 1) were lyophilized from 1,4-dioxane. 1.01 g (95%) of the title compound were obtained.

Melting Point: 93 -97°C. $[\alpha]_D$ -19.4° (C = 1.322, chloroform)

[Step e] Preparation of Benzyl 2-deoxy-4-O-diphenylphosphono-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-β-D-glucopyranoside - [Introduction of a diphenylphosphono group into the C-4 hydroxyl group and removal of the C-6 trityl group]

The product of the preceding step (0.6 g) was dissolved in a mixed solvent (3 ml) of anhydrous dichloromethane and pyridine (2 : 1), to which DMAP (0.01 g) and diphenylphosphoric acid (0.4 g) were added. The mixture was stirred over night at room temperature. Chloroform was added and the mixture was washed with 2N hydrochloric acid and with water and was then dried over sodium sulfate and concentrated in vacuo. The obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with dichloromethane were dissolved in acetone (30 ml). HBF_4 (0.03 g) was added to the mixture which was stirred for one hour at room temperature. The reaction mixture was neutralized with triethylamine and was concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with a mixture of dichloromethane and methanol were lyophilized from 1,4-dioxane. 0.426 g (71%) of the title compound were obtained.

Melting Point: 92 -93°C. $[\alpha]_D$ -17.5° (C = 1.10, chloroform).

NMR data ($CDCl_3$) δ : 3.08 (very broad t, 1H, OH), 3.48 (~d, 1H, $J_{4,5}$ ~10Hz, H-5), 3.5 -3.8 (m, 3H, H-2, H-6), 4.72 (q, 1H, $J_{3,4} = J_{4,5} = J_{4p}$, 9-10Hz, H-4), 5.50 (d, 1H, $J_{1,2}$, 8.4Hz, H-1), 5.56 (dd, 1H, $J_{2,3}$ ~10.3, $J_{3,4}$, [9.2Hz, H-3), 7.1 -7.4 (m, 15H, ph).

[Step f] Preparation of 2-Deoxy-4-O-diphenylphosphono-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucose [Removal of the C-1 benzyl group]

The product of the preceding step (0.11 g) was dissolved in methanol (20 ml), to which Pd-black (0.05 g), which had been previously reduced, was added. The mixture was stirred over night under hydrogen gas at room temperature. After the removal of the remaining catalyzers by filtration, the filtrate was washed well with methanol. The filtrate and washings were combined and concentrated in vacuo. The obtained syrups were subjected to column chromatography (Wako gel C-200) whereby the column was eluted with a mixture of dichloromethane and methanol (100:1). The title compound (0.1 g) was quantitatively obtained.

Melting Point: 68 -70°C. $[\alpha]_D$ +4.2° (C = 0.622, chloroform).

IR$_\nu$ film cm^{-1} = 3600 -3150 (OH, NH), 1740 (ester), 1660, 1540 (amide), 960 (P-O-ph), 800 -670 - (ph).

NMR data ($CDCl_3$), α:β = Ca. 2 : 1 δ : 0.75 -0.95 (m, 12H, Me), 1.0 -1.7 (m, 84H, CH_2), 2.1 -2.5 (m, 8H, CO $\underline{CH_2}$), 4.65 -4.83 (2q, 1H, H-4α,β), 5.26, (β), 5.46 (α) (2dd, 1H, H-3$_{\alpha,\beta}$), 5.33 (d, 2/3H, H-1α), 6.29, 6.83 (2d, 1H, $J_{8,1}$, 6.2Hz, $NH_{\alpha,\beta}$), 7.05 -7.4 (m, 10H, ph).

[Step g] The objective compound of Example 1 [Removal of the diphenyl group from the C-4 diphenylphosphono group]

The product of the preceding step (0.06 g) was dissolved in a mixture (50 ml) of methanol and ethanol - (1 : 1), to which Platinum oxide (0.01 g), which had been previously reduced, was added. The mixture was stirred over night at room temperature under hydrogen gas, After removing the remaining catalyzers by filtration, the reaction mixture was well washed with a mixture of chloroform and methanol (1 : 1). The filtrate and washings were combined and concentrated in vacuo. The thus obtained material was lyophilized from 1,4-dioxane. The objective compound (0.52 g) was quantitatively obtained.

Melting Point: 152 -153°C.

$[\alpha]_D$ +14° (C = 0.52, chloroform : methanol = 3 : 1)

IR ^{KBr}cm^{-1} = 3680 -2500 (OH, NH, CH), 1740 (ester), 1660, 1560 (amide).

Analysis (%) for $C_{62}H_{118}NO_{14}P$ = 1132.55

Calcd.: C, 65.75; H, 10.50; N, 1.24

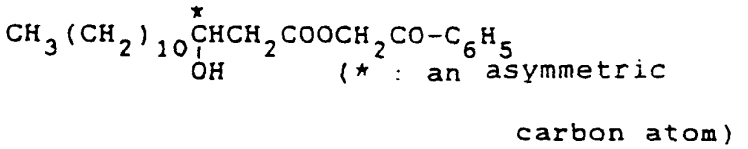
Found : C, 65.39; H, 10.67; N, 1.18

2-Deoxy-4-O-phosphono-2-(3'-tetradecanoyloxytetradecanmido)-3-O-(3'-tetradecanoyloxytetradecanoyl)-D-glucose was also prepared by the inventors in the same manner as described in Example 1 except for reacting the C-2 amino and C-3 hydroxyl groups respectively with a 3-tetradecanoyloxytetradecanoyl group (neither a rectus nor a sinister type). The compound has the following physicochemical constants: $[\alpha]_D$ +11° (C = 0.14, chloroform : methanol = 3 : 1)

Example 2: Preparation of 2-Deoxy-4-O-phosphoryl-2-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-or (3'S)-3'-hydroxytetradecanoyl]-D-glucose [Compound No. II (RR) or (SS)]

[Step a] Preparation of (3R)-or (3S)-(benzyloxymethoxy)-tetradecanoic acid [A compound to be introduced into the side chain of the C-3 hydroxyl group]

Respectively 2.3 g of (R)-or (S)-3-hydroxy-tetradecanoic acid acetophenone ester represented by the following formula:



$$CH_3(CH_2)_{10}\overset{*}{C}HCH_2COOCH_2CO-C_6H_5$$
$$OH$$

(* : an asymmetric carbon atom)

(this compound is commercially available) were dissolved in a mixture (18.4 ml) of dichloromethane and diisopropylethylamine (1 : 1).

Benzyloxymethylchloride [= C_6H_5-CH_2OCH_2Cl, 3.71 ml] was added under cooling with ice, and the mixture then stirred at room temperature. The completion of the reaction was confirmed by means of thin layer chromatography (dichloromethane : methanol = 150 : 1). Methanol was added to the reaction mixture, which was then concentrated in vacuo. The thus obtained residue was dissolved in chloroform and washed with 2N hydrochloric acid and water, dried and concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-300). The material (a R type or a S type) which was respectiveiy eluded using a mixture of hexane and ethyl acetate (15 : 1) was dissolved in acetic acid (20 ml). Zink powder (4.3 g) was added to the mixture and it was stirred over night at 50°C. The zink powder was removed by filtration and washed with dichloromethane. The filtrate and washings were combined and concentrated in vacuo. The obtained syrups were subjected to column chromatography (Wako gel C-300). The material eluted with dichloromethane or a mixture of hexane and ethyl acetate (10 : 1) was lyophilized from 1,4-dioxane. The title (3R)-or (3S)-compounds were separately obtained (together 2.2 g, 95 %).

(R)-Compound: Syrup $[\alpha]_D$ -6.7° (C = 0.924, chloroform)
Analysis (%) for $C_{22}H_{36}O_4$ = 364.51
Calcd.: C, 72.49; H, 9.96
Found : C, 72.30; H, 10.12
(S)-Compound: Syrup $[\alpha]_D$ +4.0° (C = 1.34, chloroform)
Analysis (%) for $C_{22}H_{36}O_4$
Calcd.: C, 72.49; H, 9.96
Found : C, 72.36; H, 9.89

[Step b] Preparation of (3R)-or (3S)-3-tetradecanoyloxytetradecanoic acid [the compound to be introduced into the C-2 amino group] .

The same (3R)-or (3S)-starting compounds as in the preceding step (together 2.5 g) were separately dissolved in pyridine (27 ml), to which tetradecanoic chloride (= myristoyl chloride, 2.05 g) and a very small quantity of DMAP were added. The mixture was stirred overnight at room temperature. The produced material was dissolved in acetic acid (20 ml) and zink powder (4.3 g) was added thereto. After stirring over night, the zink powder was removed by filtration and well washed with dichloromethane. The filtrate and washings were combined and concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-300). The material eluted with a mixture of hexane and ethyl acetate (10 : 1) was lyophilized from 1,4-dioxane. The title (3R)-or (3S)-compounds were obtained separately (together 2.7 g, 86 %).

(R)-Compound: Melting Point 38.5 -40°C. $[\alpha]_D$ -0.93° (C = 1.40, chloroform)
Analysis (%) for $C_{28}H_{54}O_4$ = 454.71
Calcd.: C, 73.95; H, 11.97
Found : C, 73.84; H, 12.00

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(S)-Compound: $[\alpha]_D$ +0.56° (C = 0.924, chloroform)
Analysis (%) for $C_{28}H_{54}O_4$
Calcd.: C, 73.95; H, 11.97
Found : C, 74.15; H, 11.88

[Step c] Preparation of Benzyl 2-deoxy-4,6-O-isopropylidene-2-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanamido]-β-D-glucopyranoside [Introduction of the product of the preceding Step b into the C-2 amino group].

Using the same procedure as in step a of Example 1 and employing as starting material the products (3 g) of the preceding step, the title compounds (2.2 g, 91 %; 2.1 g, 89 %) were obtained.

(R)-Compound: Melting Point 66 - 70°C. $[\alpha]_D$ -49.3° (C = 1.127, chloroform)
Analysis (%) for $C_{44}H_{75}NO_8$ = 746.05
Calcd.: C, 70.83; H, 10.13; N, 1.88
Found : C, 70.68; H, 9.99; N, 1.82
(S)-Compound: Melting Point 79 -82°C. $[\alpha]_D$ -44.9° (C = 1.20, chloroform)
Analysis (%) for $C_{44}H_{75}NO_8$
Calcd.: C, 70.83; H, 10.13; N, 1.88
Found : C, 70.60; H, 10.23; N, 1.78

[Step d] Preparation of Benzyl 3-O-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)-tetradecanoyl]-2-deoxy-4,6-O-isopropylidene-2-[(3'R)-or (3'S)-3'-tetradecanoyloxy tetradecanamido]-β-D-glucopyranoside [Introduction of the product of the foregoing step a into the C-3 hydroxyl group]

The product (0.75 g) of the preceding step was dissolved in anhydrous dichloromethane (5 ml), and the product (0.37 g) of the foregoing step b, DCC (0.5 g) and DMAP (0.08 g) were added. The mixture was stirred at room temperature. The completion of the reaction was confirmed by means of thin layer chromatography (ethyl acetate : hexane = 1 : 1). The precipitated urea was removed by filtration and washed with dichloromethane. The filtrate and washings were combined and concentrated in vacuo. The thus obtained syrups were subjected to column chromatography (Wako gel C-200). The effluents obtained with a mixture of hexane and ethyl acetate (10 : 1) were lyophilized from 1,4-dioxane. The title (3'RR)-or (3'SS)-compounds were separately obtained (together 1.08 g, 100 %).

(3'RR)-Compound: Melting Point 71 -72°C. $[\alpha]_D$ -22° (C = 0.91, chloroform)
Analysis (%) for $C_{66}H_{109}NO_{11}$ = 1092.54
Calcd.: C, 72.55; H, 10.06; N, 1.28
Found: C, 72.76; H, 10.20; N, 1.31
(3'SS)-Compound: Melting Point 38 -40°C. $[\alpha]_D$ -32.1° (C = 1.126, chloroform).
Analysis (%) for $C_{66}H_{109}NO_{11}$
Calcd.: C, 72.55; H, 10.06; N, 1.28
Found : C, 72.80; H, 10.31; N, 1.30

[Step e] Preparation of Benzyl 3-O-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)-tetradecanoyl]-2-deoxy-2-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanamido]-β-D-glucopyranosid [Removal of the isopropylidene group]

Using the same procedure as in step c of Example 1 and employing as starting material the (3'RR)-or (3'SS)-products (0.87 g, 0.8 g, respectively) of the preceding step, the title compounds were separately obtained (0.73 g, 87 %; 0.66 g, 85%).

(3'RR)-Compound: Melting Point 100 -101.5°C. $[\alpha]_D$-35.9° (C = 0.754, chloroform)
(3'SS)-Compound: Melting Point 94 -96°C. $[\alpha]_D$ -14° (C = 1.213, chloroform)

[Step f] Preparation of Benzyl 3-O-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)-tetradecanoyl]-2-deoxy-2-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanamido]-6-O-trityl-β-D-glucopyranoside [Introduction of a trityl group into the C-6 hydroxyl group]

Using the same procedure as in step d of Example 1 and employing as starting material the (3'RR)-or -(3'SS)-products (0.68 g, 0.7 g, respectively) of the preceding step, the title compounds were separately obtained (0.78 g, 93 %; 0.77 g, 90 %).

(3'RR)-Compound: $[\alpha]_D$ -31.2° (C = 0.902, chloroform)
(3'SS)-Compound: Melting Point 70 -72°C. $[\alpha]_D$ -17° (C = 0.87, chloroform)

[Step g] Preparation of Benzyl 3-O-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)-tetradecanoyl]-2-deoxy-4-O-diphenylphosphono-2-[(3'R)-or (3'S)-3'-tetradecanoyloxy tetradecanamido]-β-D-glucopyranoside [Introduction of a diphenylphosphono group into the C-4 hydroxyl group and removal of the C-6 trityl group]

Using the same procedure as in step e of Example 1 and employing as starting material the products -(0.55 g, 0.55 g, respectively) of the preceding step, the title compounds (0.374 g, 68 %; 0.363 g, 66 %) were separately obtained.

(3'RR)-Compound: Melting Point 69.5 -70.5°C. $[\alpha]_D$ -14.5° (C = 0.724, chloroform)
(3'SS)-Compound: Melting Point 67 -70°C. $[\alpha]_D$ -16.3° (C = 1.02, chloroform)

[Step h] Preparation of 2-deoxy-4-O-diphenylphosphono-3-O-[(3'R)-or (3'S)-3'-hydroxytetradecanoyl]-2-[-(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanamido]-D-glucose [Removal of the C-1 benzyl group and removal of a benzyloxymethyl group from the side chain on the C-3 substituent]

Using the same procedure as in step f of Example 1 and employing as starting material the products - (0.158 g, 0.16 g, respectively) of the preceding step, the title compounds (0.128 g, 97 %; 0.14 g, 100 %) were separately obtained.

(3'RR)-Compound: Melting Point 87 -88°C. $[\alpha]_D$ -2.1° (C = 1.17, chloroform)
(3'SS)-Compound: Melting Point 64.5 -65°C. $[\alpha]_D$ +9.5° (C = 0.786, chloroform)

[Step i] Preparation of the objective compound of Example 2 [Removal of the diphenyl group from the C-4 diphenylphosphono group]

Using the same procedure as in step g of Example 1 and employing as starting material the products - (0.128 g, 0.14 g, respectively) of the preceding step, the final objective compounds were separately obtained.

(3'RR)-Compound: Melting Point 172 -174°C. $[\alpha]_D$ +12.8° (C = 0.97, Chloroform : methanol = 3 : 1)
IR$_\nu^{KBr}$ cm^{-1} = 3680 -2500 (OH, NH, CH), 1740, 1720 (ester), 1645, 1650 (amide).
Analysis (%) for $C_{48}H_{92}NO_{13}P$ = 922.21
Calcd.: C, 62.51; H, 10.06; N, 1.52
Found : C, 62.85; H, 9.93; N, 1.60
(3'SS)-Compound: Melting Point 156 -158°C. $[\alpha]_D$ +17.2° (C = 0.571, chloroform : methanol = 3 : 1)
IR$_\nu^{KBr}$ cm^{-1} = 3680 -2500 (OH, NH, CH), 1740, 1720 ester), 1655, 1550 (amide).
Analysis (%) for $C_{48}H_{92}NO_{13}P$
Calcd.: C, 62.51; H, 10.06; N, 1.52
Found : C, 62.30; H, 10.26; N, 1.35

2-Deoxy-3-O-(3'-hydroxytetradecanoyl)-4-O-phosphoryl -2-(3'-tetradecanoyloxytetradenamido)-D-glucose was also prepared in the same manner as carried out through the steps from 'a' to 'i' in Example 2 but employing materials which were neither the rectus type nor the sinister type. The compound exhibits the following physico-chemical constants:

$[\alpha]_D$ +8.76° (C = 0.616, chloroform)
UR$_\nu$ Nujol mull cm^{-1} = 3600 -3200 (OH, NH), 1720 (ester), 1640, 1540 (amide).
Analysis (%) for $C_{48}H_{92}NO_{13}P$
Calcd.: C, 62.51; H, 10.06; N, 1.52
Found : C, 62.39; H, 10.23; N, 1.52

Example 3: Preparation of 2-Deoxy-2-[(3'R)-or (3'S)-3'-hydroxytetradecanamido]-3-O-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanoyl]-4-O-phosphoryl-D-glucose

[Step a] Preparation of Benzyl 2-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)tetradecanamido]-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside [Reaction with the C-2 amino group]

Following step c of Example 2 but employing the product of step a of Example 2 for the product of step b of Example 2, the title compounds were obtained.

(3'R)-Compound: Melting Point 109 -110°C, $[\alpha]_D$ -56.5° (C = 0.66, chloroform), Yield 80.2 %
(3'S)-Compound: Melting Point 67 -70°C, $[\alpha]_D$ -49.7° (C = 0.561, chloroform), Yield 84 %

[Step b] Preparation of Benzyl 2-[(3'R)-or (3'S)-3'-(benzyloxymethoxy)tetradecanamido]-2-deoxy-4,6-O-isopropylidene-3-O-[(3'R)-or (3'S)-3'-tetradecanoyloxytetradecanoyl]-β-D-glucopyranoside [Introduction into C-3 hydroxyl group]

Following step d of Example 2 but employing the product of step a of Example 2 for the product of step b of Example 2, the title compounds were obtained.

(3'RR)-Compound: Melting Point 70 -72°C, $[\alpha]_D$ -24.6° (C = 1.21, chloroform), Yield 92 %.
(3'SS)-Compound: Melting point 39 -40°C, $[\alpha]_D$ -30.1° (C = 1.18, chloroform), Yield 100 %.

[Steps c -g] Preparation of the objective compounds of Example 3

Following steps e -i of Example 2 the product of the preceding step was subjected to the following chemical reactions : (c) Removal of the C-4,6-O-isopropylidene group, (d) Introduction of a trityl group into the C-6 OH group, (e) Introduction of a diphenylphosphono group into the C-4 OH group and removal of the C-6 trityl group, (f) Removal of the C-1 benzyl group and the benzyloxymethyl group from the substituent connected with the C-2 amino group and (g) Removal of the diphenyl group from the substituent connected with the C-4 OH group. Thus, the title compounds w re separately obtained.

(3'RR)-Compound: Melting Point 157 -159°C, $[\alpha]_D$ +13.7° (C = 0.512, chloroform : methanol = 3 : 1)
IRν KBr max cm^{-1} = 3680 -2500 (OH,NH,CH), 1735, 1720 (ester), 1640, 1560 (amide)
(3'SS)-Compound: Melting Point 154 -155°C, $[\alpha]_D$ +18.4° (C = 0.896, chloroform : methanol = 3 : 1)

2-Deoxy-2-(3'-hydroxytetradecanamido)-3-O-(3'-tetradecanoyloxytetradecanoyl)-4-O-phosphoryl-D-glucose was also prepared following the steps from 'a' to 'g' of Example 3 but employing materials which were neither the rectus nor the sinister type. This compound exhibits the following physico-chemical constants:

$[\alpha]_D$ +7.69° (C = 0.442, chloroform)
IRν Nujol max cm^{-1} = 3600 -3100 (OH, NH), 1720 (ester), 1630, 1540 (amide)
Analysis (%) for $C_{48}H_{92}NO_{13}$ = 922.21
Calcd.: C, 62.51; H, 10.05; N, 1.52
Found : C, 62.44; H, 10.18; N, 1.50

Example 4: Preparation of 2-Deoxy-4-O-phosphoryl-2-tetradecanamido-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucopyranose

[Step a] Preparation of Benzyl 2-deoxy-4,6-O-isopropylidene-2-tetradecanamido-β-D-glucopyranoside [Introduction of a tetradecanoyl group into C-2 amino group]

Following step a of Example 1 but employing tetradecanoic acid for (3'R)-3'-tetradecanoyloxytetradecanoic acid, the title compound was obtained in a yield of 88.5 %.

[Step b] Preparation of Benzyl 2-deoxy-2-tetradecanamido-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-β-D-glucopyranoside [Introduction of a (3R)-3-tetradecanoyloxytetradecanoyl group into the C-3 OH group and removal of the 4,6-O-isopropylidene group]

The product (800 mg) of the preceding step was dissolved in dichloromethane (8 ml), and (3R)-3-tetradecanoyloxytetradecanoic acid (700 mg), DMAP (95 mg) and DCC (400 mg) were added. The mixture was left for 8 hours at room temperature. The completion of the reaction was confirmed by means of thin layer chromatography. The precipitated urea was removed by filtration and the filtrate was concentrated in vacuo. The thus obtained product was dissolved in a mixture of 90 % acetic acid, dichloromethane and methanol and the solution was stirred at 50° C. After confirming the completion of the reaction, the reaction mixture was concentrated in vacuo and the residue was subjected to column chromatograohy (Wako gel C-300). Using as eluent (a) dichloromethane and (b) a mixture of dichloromethane and methanol (250 : 1), the title compound (480 mg, 49 %) was obtained with solvent (b).

$[\alpha]_D$ -21.48° (C = 3.453, chloroform)

[Steps c -f] Preparation of the objective compound of Example 4

Following steps d -g of Example 1 the product of the preceding step was subjected to the chemical reactions of: (c) Tritylation of the C-6 OH group, (d) Diphenylphosphorylation of the C-4 OH groups and removal of the trityl group, (e) Removal of the C-1 benzyl group and (f) Removal of the diphenyl group from the substituent connected to the C-4 group. Thus, the title objective compound was obtained.

Melting Point 150 -151°C.
IRν KBr max cm^{-1} = 3450 (OH, NH), 2960, 2870 (CH_2, CH_3), 1740 (ester), 1650, 1560 (amide)

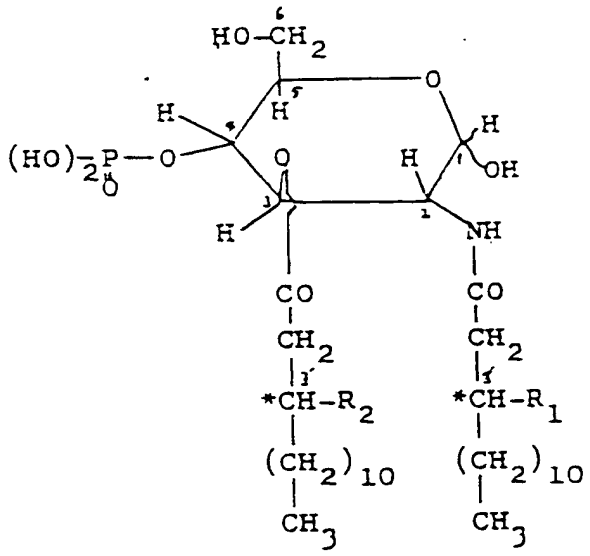
The study of the stereoisomers teaches that a rectus configuration and a sinister configuration, which are formed with an asymmetric carbon atom as a centre, stand in a diastereomer relation to each other, which is not identical with an optical antipode relation between a dextro and a levo type . So, as seen in the examples of this specification, a mother compound and its rectus and sinister compounds exhibit different physico-chemical properties such as melting point, the angle of optical rotation and solubility, and consequently, exhibit different biological and immunological activities with each other.

This is why the inventors were investigating the stereoisomers of some derivatives of the nonreducing monosaccharide subunit of lipid A which have so far been synthesized. They were identified to have certain interesting biological and immunological properties.

To state more concretely, the compounds of this invention are expected to exhibit definite effects for proclotting the enzyme of horseshoe crab, inducing interferon-and tumor-necrosis factors, furthermore, they act as mitogens for polyclonal B cells and as adjuvant.

Claims

1. Compounds having the general formula I



[I]

wherein R_1 is a hydrogen atom, hydroxyl group or $-O-CO-(CH_2)_{12}-CH_3$ and R_2 represents a hydroxyl group or $-O-CO-(CH_2)_{12}-CH_3$, and stereoisomers thereof.

2. 2-Deoxy-4-O-phosphono-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucose

3. 2-Deoxy-4-O-phosphono-2-(3'-tetradecanoyloxytetradecanamido)-3-O-(3'-hydroxytetradecanoyl)-D-glucose

4. 2-Deoxy-4-O-phosphono-2-[(3'R)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'R)-3'-hydroxytetradecanoyl]-D-glucose

5. 2-Deoxy-4-O-phosphono-2-[(3'S)-3'-tetradecanoyloxytetradecanamido]-3-O-[(3'S)-3'-hydroxytetradecanoyl]-D-glucose

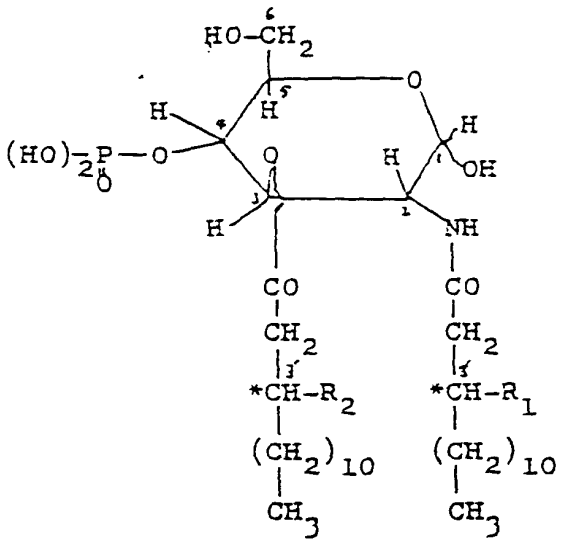
6. 2-Deoxy-4-O-phosphono-2-(3'-hydroxytetradecanamido]-3-O-(3'-tetradecanoyloxytetradecanoyl]-D-glucose

7. 2-Deoxy-4-O-phosphono-2-[(3'R)-3'-hydroxytetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucose

8. 2-Deoxy-4-O-phosphono-2-[(3'S)-3'-hydroxytetradecanamido]-3-O-[(3'S)-3'-tetradecanoyloxytetradecanoyl]-D-glucose

9. 2-Deoxy-4-O-phosphono-2-tetradecanamido]-3-O-[(3'R)-3'-tetradecanoyloxytetradecanoyl]-D-glucose

10. A process for preparing the compounds of the general formula I



[I]

characterized by

(a) the reaction of a 3'-O-(substituted or non substituted)-tetradecanoyl radical with the amino group in the C-2-position of benzyl 2-amino-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside in the presence of DCC,

(b) reaction of the same radical with the hydroxyl group in the C-3-position in the presence of DCC and/or DMAP,

(c) the removal of the protective groups from the C-4-and C-6-positions,

(d) tritylation of the C-6-hydroxyl group,

(e) introduction of a diphenylphosphono group in the C-4-position and removal of the C-6-tritylgroup,

(f) removal of the C-1-benzyl group,

(g) optional removal of the benzyloxymethyl group from the C-3-side chain, and

(h) removal of the diphenyl group from the C-4-position.

11. The use of the compounds according to claims 1 to 9 for proclotting the enzyme of horseshoe crab, inducing interferon-and tumor-necrosis factors, as mitogens for polyclonal B cells and as adjuvant.